Textbook of CLINICAL EMBRYOLOGY

EDITED BY **Kevin Coward** and **Dagan Wells**

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Foreword

It is a pleasure to pen the Foreword to this *Textbook of Clinical Embryology*. As someone who was in at the 'ground floor', it has always surprised me that it has taken so long to produce such a volume! After all, the basis for the body of knowledge produced here was first established in the 1940s and 1950s with the accumulation of the Carnegie collection of human embryos (Hertig *et al.*, 1956; Rock and Menkin, 1944; Rock and Hertig, 1948). However, the main stimulus to the explosive growth in studies on human embryos can be dated to a 1965 *Lancet* paper by Bob Edwards (1965), which described the maturation of human eggs*in vitro*. This paper was based on research spanning the previous ten years, during which time Bob had made many significant discoveries in developmental genetics, immunological contraception and embryonic stem cells, as well as in oocyte maturation – as witnessed in his 56 papers published by 1965 (Gardner and Johnson, 2011). However, his 1965 *Lancet* paper was a landmark trigger in that its Discussion set out the course for the next 20 years of what would become known as Assisted Reproduction. It also set the scene for his following papers proving the principle of PGD (Gardner and Edwards, 1968), the demonstration of IVF (Edwards *et al.*, 1969), and the development of morulae and blastocysts *in vitro* (Edwards *et al.*, 1970; Steptoe *et al.*, 1971). These papers made human embryos available for the first time in sufficient numbers for their study scientifically. They also brought to the fore a whole new set of ethical, legal and political questions about the status of the human embryo, how it should be treated and what control should be exercised over it – moving it from science fiction to science fact (Theodosiou and Johnson, 2011). Bob was at the forefront of public debate on these issues too, early key papers being Edwards and Sharpe (1971) and Edwards (1974).

However, although Bob provided the vision, the inspiration and much of the energy for driving this field forwards, progress would not have been achieved without Patrick Steptoe. Bob originally believed that *in-vitro* matured oocytes from ovarian biopsies would be suitable for producing human embryos, and his motivation for contacting Patrick and initiating their collaboration was that Bob thought that Patrick could solve the sperm capacitation problem with which he had been wrestling since 1965 (Johnson, 2011), and which was, in fact, resolved in 1968 by the use of Bavister's medium (Bavister, 1969). However, towards the end of 1968 Bob became less sure that the *in-vitro* matured eggs would produce viable embryos, despite their chromosomal maturity, and so he and Patrick turned to laparascopic recovery of mature ovarian follicle eggs (Steptoe and Edwards, 1970). Patrick was a major pioneer in his own right, although as underappreciated at the time as was Bob (Johnson *et al.*, 2010). His book *Laparoscopy in Gynaecology* (Steptoe, 1967) is to keyhole surgery what Bob's *Lancet* paper is to ART. These two professional outcasts formed a powerful partnership, known around Bourn Hall in later years as 'Steppie and the Boss'.

There is a third player who often gets overlooked but whom it is particularly important to acknowledge in this book intended for ART practitioners, and that is Jean Purdy. Jean joined Bob in 1968 as his technician, one of her attractions being her nursing qualification, a sign of the increasing importance that his forays into use of clinical material was assuming. She worked with him and Patrick until her early death aged 39 in 1985 (Edwards and Steptoe, 1985). Jean was as hard-working and dedicated as both Steppie and the Boss, and had two attributes that were of key importance for the success of their partnership. Perhaps the most important, as has become clear from a recent analysis of a newly discovered set of Oldham notes and notebooks that Kay Elder and I are working through, is her organizational role – for it was Jean who methodically took all the notes made by Bob and Patrick on scraps of paper and entered, cross-checked and summarized

Fig 0.1 Bob Edwards (1925–2013), Jean Purdy (1946–85) and Patrick Steptoe (1913–88) at Bourn Hall in 1981 (courtesy Bourn Hall Clinic).

them in the notebooks to give the detailed records on which they based their work over the period from 1969 to 1978 (and which we intend soon to publish). Bob and Patrick clearly relied on Jean to undertake this difficult and demanding task, which she appears to have performed meticulously. Less easy to evaluate is her role as the 'oil' in the relationship between these two strong-willed and determined men, between whom (despite, and perhaps even because of, their assigned roles as outcasts) sparks must have flown at times, both being under a lot of pressure – both internal and from outside.

Sadly, neither Patrick nor Jean were alive to share in the award or the joy of the Nobel Prize that went to Bob in 2010, and even Bob by then was too ill to attend in person, although delighted at the eventual recognition some 45 years after that *Lancet* paper that set the whole of ART in train. Were Bob alive today, I am sure that he would have been delighted to write this Foreword – although it would have taken a very different form – generous about the book's scope and content but wagging that finger gently and with his rueful smile (that says 'it pains me to say this') at what he thought was wrong and missing!

Professor Martin Johnson

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Preface

In the three decades since the birth of Louise Brown, the first child conceived using in-vitro fertilization (IVF), the field of clinical embryology has undergone remarkable growth and evolution. The discipline has come to embrace a wide-variety of specialized laboratory techniques, collectively falling under the umbrella-term *assisted reproductive technology* (ART). Worldwide, over 1 million ART cycles are carried out each year and over 5 million babies are estimated to have been born as a direct consequence. There is no doubt that ART represents one of the most successful interventions in any field of medicine. It has radically altered the way in which most forms of infertility are treated and bought hope to millions of infertile and sub-fertile couples around the world. However, it must be acknowledged that, despite the obvious successes, significant technical challenges still remain and scientific knowledge in some areas of clinical embryology is limited.

With the expansion of ART has come an ever greater emphasis on quality assurance and, in some countries, an increase in the extent to which treatments are overseen by independent or governmental bodies. In order to ensure that patients consistently receive optimal clinical care and the best chances of conception, meticulous training of new personnel in theoretical knowledge as well as practical skills is critical. However, it is equally vital that established doctors, nurses and embryologists constantly refresh their store of knowledge, keeping abreast of changes in the regulatory environment and understanding the benefits and limitations of new technologies – what is proven and what is, at least for the time being, hypothesis or conjecture.

This textbook was inspired by the M.Sc. in Clinical Embryology (University of Oxford), an intensive one-year residential course that aims to motivate future leaders in clinical embryology and reproductive medicine, inspiring them to investigate the molecular and physiological mechanisms underlying human infertility. This course is now in its fifth successful year and continues to attract global interest, with student representation from 28 countries thus far. This textbook has been compiled by senior academic or clinical staff associated with the M.Sc. course, and aims to present a holistic approach to the treatment of human infertility and the biological mechanisms involved.

We would like to extend our special thanks to Nick Dunton at Cambridge University Press (CUP) for thoughtful and insightful discussion during the early phases of this project, and, above all, his patience during the extended period thereafter. We would also like to thank the following staff at CUP for their help and assistance during the copy-editing and production process: Jodie Hodgson, Lucy Edwards, Christopher Miller and Jane Seakins. Special thanks to Karen Verde at Green Pelican Editorial Services (NJ, USA) for copy-editing this large body of work in such a rapid manner. Special thanks also go to Mr Hamnah Bhatti (University of Oxford Medical School) for creating some of the illustrations provided in Chapters 8 and 32. Several members of the Nuffield Department of Obstetrics and Gynaecology (University of Oxford) provided key support, including Celine Jones, Junaid Kashir and Siti Nornadhirah Amdani. Finally, we would like to thank all of our authors for their support, dedication and patience.

We dedicate this textbook to the ever-lasting legacy of Professor Sir Robert Edwards.

Kevin Coward and Dagan Wells

Chapter 1

Sexual reproduction: an overview

Suzannah A. Williams

Introduction

Reproduction is the production of offspring, propagating genes into the next generation, and exists in many forms within the animal kingdom. Each of these different strategies has advantages and disadvantages, but all strategies have evolved as the optimum for a particular species in a particular niche. Sexual reproduction, as opposed to asexual reproduction, in the majority of cases involves the *recombination* of DNA to result in the generation of unique individuals. Of these individuals, some will be better adapted to exist in the surrounding environment than others, and these better suited individuals are most likely to be more successful. Therefore, this process of evolution not only results in the success of the fittest but also leads to intense competition for the best mate to produce the 'best' next generation.

For successful reproduction in mammals, i.e. the production of new viable offspring, there are many different stages that are essential not only in function but also timing. These stages include the production of functional gametes, appropriate behaviour to ensure the released gametes interact, a suitable environment for implantation and subsequent embryo development, birth to occur into a suitable environment and also for appropriate lactation to ensure the newborn is adequately provided for. Failure at any of these earlier stages can result in infertility ultimately failing to produce viable offspring, and in the worst case, threatens the life of the mother and of the fetus or newborn(s). Understanding how each of these events is regulated is critical for furthering our ability to *influence* these processes. This is critical not only to assist people who are unable to conceive naturally to have children, but also for other purposes such as to aid fertility in endangered species and to maximize reproduction for food production.

Although the focus in this textbook is on the mechanisms of reproduction in humans, there are numerous insights to be drawn from investigating reproductive strategies in other species.

Gamete generation and selection

The production of gametes for reproduction requires, in the case of the male, sperm that are mobile and functional, and in the female, the ovulation of an egg that is effectively the best of all those developing in the ovary.

In the selection of the 'best' gamete(s) there is enormous wastage of both male and female gametes which occurs at different stages in their generation. In males, selection occurs primarily after ejaculation. Millions of spermatozoa are produced by each male on a daily basis, calculated at 1000 per second in the human [1], however the number of sperm that actually reach the site of fertilization is understood to be remarkably low, with only one spermatozoa actually required for fertilization. Therefore, the vast majority of male gametes are unsuccessful in the pursuit of reproduction. Whereas in women, selection occurs in the ovary by a variety of mechanisms with several follicles growing but ultimately only one egg is ovulated in the vast majority of cases.

While we understand something of the mechanisms that regulate the number of eggs that are ovulated in humans (discussed further in Chapters 4–6) we have very little understanding of how ovulation rate is regulated between species. This is key to fully understand ovarian function and fertility regulation in all species including humans. Current techniques for obtaining large numbers of eggs in women undergoing IVF require high doses of hormones and although they are effective in attaining the objective, the administration of these hormones poses a significant risk to the

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woman, namely ovarian hyperstimulation syndrome [2] (discussed further in Chapters 25 and 29).

It is not yet known how the egg that is selected for ovulation in a normal cycle differs to those that undergo atresia and die. Ovarian stimulation in women allows a whole cohort of follicles to develop and multiple eggs to be ovulated, and yet we have little knowledge about which eggs should be used first as the 'best' eggs for assisted reproduction. Therefore, furthering our understanding of ovulation rate and the mechanisms that regulate it are critical to developing more natural ways of obtaining eggs and to enhancing our selection of the best eggs.

It is clear that there is considerable wastage of potential female gametes, primarily due to the considerable numbers of oocytes that are generated and develop compared to the very low number ovulated. Indeed, females generate approximately 7 million primordial germ cells [3] (discussed further in Chapter 6) and ovulate around 400 before undergoing menopause at approximately 50 years of age in Western women. An alternative way to think about it is that to select the finest, you need to have a heterogeneous pool to select from. Perhaps, rather than perceive this loss of oocytes as wastage, we should view it as selection. Since all of the oocytes within the pool will vary to some extent based for example on location in the ovary, proximity during development to other follicles, vasculature, it is possible that the 'best' oocyte to be selected within a pool of oocytes varies depending on a woman's age or available nutrition. Therefore the generation of a pool of oocytes for each cycle is required so that the most appropriate can be selected. Sperm selection also exists. In addition to sperm selection within the female reproductive tract where the sperm that fertilizes has good forward motility and is headed in the right direction at the outset, there is good evidence for elimination of many genetically or otherwise abnormal sperm via cell cycle checkpoints and apoptosis. A sperm chemoattractant has been postulated for many years. Anyone who has added sperm to eggs in culture will have observed that an overwhelming number of sperm bind to the eggs. Recently progesterone has been found to have sperm-attracting properties [4] although this may not be the only factor involved.

The distance that sperm need to cover to reach the fertilization site in the fallopian tube is considerable, taking into account the size of the sperm. For many years the sperm was considered to be propelled forward by the tail moving in a side-to-side whiplash motion; however we now know that the tail drives the sperm forward by a corkscrew action. Calculations of the time it takes sperm to travel the distance have revealed that other mechanisms exist to carry the sperm to the fertilization site, including fluid flow to the oviduct. However, if the sperm are pulled towards the 'wrong' oviduct, i.e. the one that does not contain an ovulated egg, then these sperm are effectively out of the race.

Other species have evolved novel mechanisms for sperm transport in the female tract. For instance the sperm head of the common wood mouse is hook-shaped, and these hooks attach to one another forming trains (Fig. 1.1a). The hook-shaped head is a characteristic of rodent sperm and the specific shape of this hook affects how the heads are able to join and interact. These sperm trains have an increased speed compared to single sperm. Furthermore, these trains also contain sacrificial sperm, which sacrifice their acrosome to join the train, thereby rending them unable to fertilize the egg [5].

Spermatozoa are produced in the testes which are external to the body cavity in most mammals. Temperature regulation is critical to the production of functional sperm in humans. The question arises as to why sperm production in mammals requires a lower temperature in some species. It is possible that it is an evolutionary advantage for sperm to die at body temperature and therefore with each new fertilization, new sperm are required, ensuring that for each conception, the sperm that fertilizes is from the current fittest sire. Alternately, females who are unwell with an elevated temperature will enhance sperm death, thus aiding the prevention of pregnancy in women who are unwell.

However, external testes do not exist in all mammals, and indeed testes temperature is not decreased in all mammals. Conversely, internal testes do not necessitate that the testes temperature is the same as the rest of the body. Dolphins have internal testes and yet the temperature of the testes is maintained lower than body temperature. This is achieved by circulating the blood that comes directly from the fins at the extremities, which is cooler, directly to the testes, thus maintaining the testes at a lower temperature.

Spermatozoa are produced in the testes from puberty till death, ensuring there is a continuous supply throughout the reproductive life of mammals. Therefore men can continue to reproduce late into their dotage. The oldest father on record is an

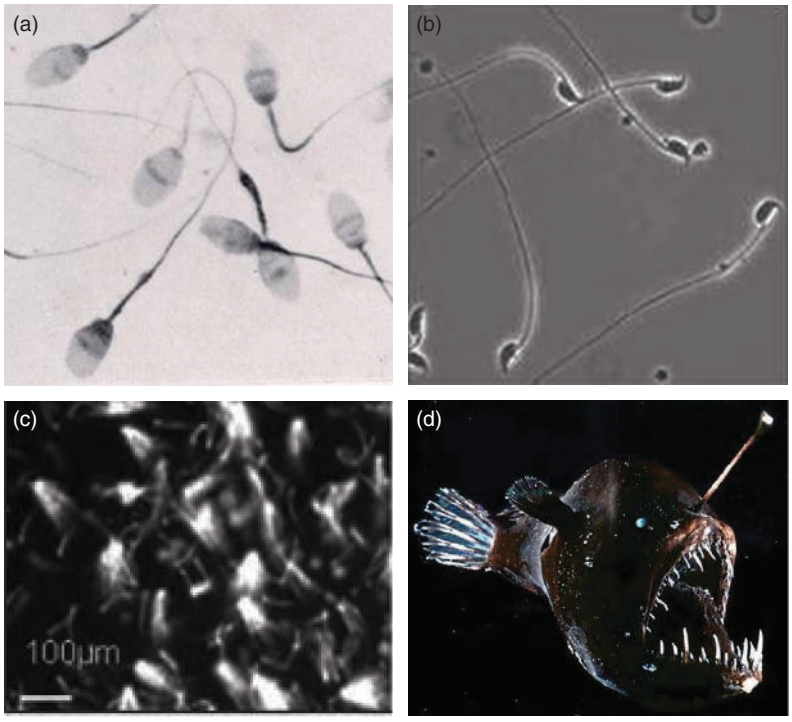

Figure 1.1 Male gametes. (a) human sperm; (b) mouse sperm; (c) wood mouse sperm trains; (d) angler fish.

Australian who fathered his last child at 92 years of age. This is in direct contrast to the limited number of eggs that exist in females (discussed further in Chapter 6). However, although sperm production is continuous, sperm production and quality are known to decline as men age [6].

Social and behavioural gamete selection

The female is the one, in most species, that carries the offspring, nurturing their development until finally giving birth, and thus it is the female that provides the vast majority of the investment in the production of young. Therefore, it is in her interest to ensure that her investment is for an offspring that has the best chance of survival, i.e. has the best genes. Thus it is in the female's best interest to ensure that she mates with the 'best' male. There are some exceptions to female pregnancy, notably seahorses, where the male carries the eggs attached to his abdomen for the duration of gestation and gives birth to numerous miniature seahorses. The 'best' criteria for a mate vary with species depending on the reproductive strategy employed. Therefore, since in most species it is the female that has the greater investment, sperm selection is the focus of social and behavioural gamete selection.

A variety of successful strategies exist to achieve a reliable source of spermatozoa. One option is a monogamous partnership with a tried and tested male. This ensures reliable functional sperm are available on demand. Furthermore, a male that is making a large investment into the offspring has greater interest in supporting their development.

Angler fish, which live at considerable depth in a very barren environment, have evolved an unusual strategy to ensure a reliable supply of sperm. For many years only female Angler fish were caught, which intrigued scientists as to the reproductive strategy employed. However, a number of females had a small but noticeable bulge on a part of their abdomen, and only when this was analysed did it become apparent that this appendage formed the remainder of the male's body. Upon encountering a female, a male Angler fish bites into the side of the female and becomes permanently attached (Fig. 1.1). The male's body atrophies and nutritional support for the remaining tissue is provided by the female's body. Sperm are released into the female Angler fish as a result of hormonal stimulation by the female.

However, this of course means that the female's choice of mate is unchangeable after the male has attached to the female. Therefore, although there is a continual supply of male gametes, there is no ability for the female to select the strongest male to supply the fittest sperm, a system that has evolved in many species. This particular strategy is fascinating, not only in its own right, but it can also potentially reveal insights into how foreign tissue can be accepted by a host more generally.

The females of some species, including many birds and reptiles, have evolved a reproductive tract with the capability of storing sperm to ensure a constant supply. The female tract contains crypts where sperm can be stored for a considerable length of time. Gould's wattled bats mate in autumn and store the sperm through hibernation until fertilization the following spring. Turtles can store sperm for 4 years and snakes have been known to store sperm for up to 7 years. Understanding the mechanisms involved that enable sperm to be stored at body temperature for such prolonged periods of time without any ill effects would clearly be an advantage to storing sperm for use in breeding programmes and for in vitro fertilization (IVF). Furthermore, additional insight would be gained by understanding not only how these specialized cells exist for this long period of time, but also how they are unaffected by increased temperature. Eliminating the need for cryopreservation for storing sperm would clearly be a great advantage for many aspects of reproductive biology.

In humans, a reliable source of spermatozoa for procreation is achieved by the existence of monogamous relationships. This is however an unusual circumstance in the animal world, where monogamous relationships are not very common. Even in species that appear to be monogamous, genetic testing of offspring and parents has revealed that many offspring are actually fathered by a different male. In this context, evolutionarily it might be advantageous to bring up offspring with a tried and tested partner from previous years; however, this male may not be the fittest male available and therefore mating with one deemed fitter by the female is clearly the way to obtain the best genetics for the offspring.

In most species, partner choice is influenced by perceived fitness which has many guises. It is most easily characterized in non-humans where the determinants appear much less complex and have been documented in many species from multiple genres. The goal in choosing the fittest mate is to ensure the offspring are given the best opportunity genetically to compete with the fittest of their generation. However, markers of fitness in different species can be remarkably obscure to the human eye. Some of the more obvious, for example, large antlers for fighting to establish male hierarchy, can be readily understood. We can also appreciate the song voice of various song birds. Whereas the long expansive plumes of the peacock are hard to understand as a mark of function but as a display to differentiate between males, it is understandable. Therefore, the 'fittest' male is not necessarily the fittest to survive the environment but may be in possession of the best genes to ensure their offspring also possess desirable partner traits and thus have the greatest chance of mating.

In contrast, partner selection in humans is extremely complex. Unlike other primates including mountain gorillas, where the dominant male is the strongest male, we have established a social structure with less aggressive principals in an evolved society and therefore strong, large males are not necessarily the optimum choice. Intelligence and a 'sense of humour' are also key factors in human mate choice [7]. Although since studies indicate that human mate choice is also dependent on an individual's specific major histocompatibility complex (MHC; important for immunity) as detected by body odour, this indicates that a primitive and subconscious aspect still exists for human mate choice. Furthermore, one of the most intriguing developments in human partner choice in the developed world is that, unlike all other primates and the majority of mammals, females now also 'exhibit' to compete for partners. Women are no longer the choosers of their mate but are also being chosen.

Fertilization

One question is why sperm binding is species-specific if it occurs within the reproductive tract of sexually reproducing species? The answer is that it is most likely a remnant from our early ancestry when fertilization occurred externally and has not been lost. However the exact mechanisms that regulate sperm binding to the egg zona pellucida in mammals have yet to be elucidated. There is considerable controversy in the field, with numerous hypotheses based on clear and convincing data, albeit conflicting [8–10] (this is discussed further in Chapter 10).

Embryo development and gestation

Preimplantation embryos generated during assisted reproduction that are surplus can be stored for further reproductive cycles. Currently this requires cryopreservation; however this does result in a degree of embryo damage and loss. Therefore, since these embryos are extremely precious, developing new methods to improve viability of preserved embryos would be advantageous. For instance, a number of marsupials, including the tammar wallaby, generate a blastocyst which remains quiescent in the female's reproductive tract for almost a year until the environment is once again optimal for reproduction [11]. This blastocyst is generated to enable the tammar wallaby to rapidly resume pregnancy if the existing offspring dies. Understanding the mechanisms that can maintain a viable blastocyst at this stage for this long period of time would of course be of great use clinically in the preservation of blastocysts, as this would prevent loss during the cryopreservation procedure.

One of the most interesting and unexpected discoveries in recent years is that mothers often retain a small number of cells from the fetus they have carried. Therefore mothers are effectively chimaeras. A high proportion of fetal cells in mothers have been linked to an increased incidence of autoimmune disease [12]. Understanding the mechanisms of not only how these cells cross the placenta but also how they contribute to the onset of autoimmune disease is a field of active scientific research.

Reproductive strategies

Mammals exhibit a variety of options for the development of offspring ranging from almost embryonic to fully formed (Fig. 1.2). Offspring born to marsupials reflect the least developed infants or newborns. Kangaroo offspring greet the world a mere 2 cm long, blind and hairless newborn (newborns this undeveloped are known as altricial). Humans are also altricial, being unable to care for themselves and relying entirely on their parents for all their requirements. This is in extreme contrast to precocial guinea pigs which are born fully formed and mobile after 6 weeks' gestation. Humans invest a great deal into their offspring, with each baby born representing significant investment and also requiring considerable future input and investment. Human offspring require many years of care and nurturing. Many mammals choose to invest in a number of offspring as opposed to focusing on raising a singleton. Altricial offspring are usually a characteristic of larger litters, however, as observed for kangaroos and humans, this is not a universal trend.

The newborn kangaroo has to make its way squirming and wriggling up the mother's stomach to the lip of her pouch into which it descends, attaches to a nipple and remains there for the next 6 months. Despite being born in an almost embryonic form, the newborn kangaroo achieves this feat unaided. Interestingly, although human offspring are born requiring considerable care and attention, if left to

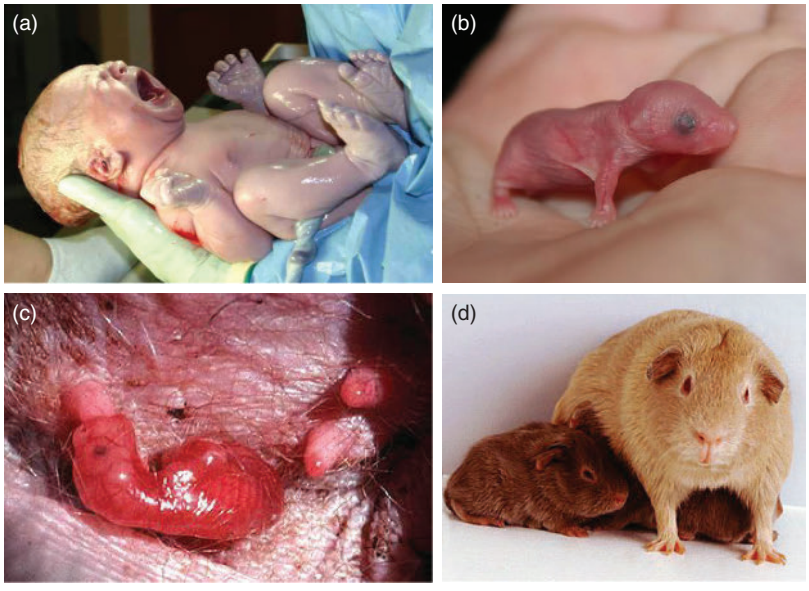

Figure 1.2 Newborn development of (a) the altricial human; (b) mouse; (c) kangaroo; and (d) the precocial offspring of the guinea pig.

their own devices after birth, they will, of their own accord, make their way up their mother's stomach to the breast for their first feed (personal communication, Professor Peter Hartmann, University of Western Australia).

Young kangaroos suckle for up to a year and during this time the composition of the milk changes from carbohydrate-rich to fat-rich milk. Other species employ different strategies and suckle their young for a considerably shorter period of time. Hooded seals suckle their young for a mere 4 days with milk containing 60% fat; as opposed to 4% in cattle and humans. During this time the pup doubles in size, generating vast reserves of blubber [13]. The fur seal, however, adopts a different strategy where pup feeding is intermittent [14]. The pup is fed for a number of days and then is abandoned for up to 4 weeks when the mother leaves the pup to forage for herself before returning to resume feeding. Interestingly, unlike humans, lactation in this species can be turned off and then on again without any apparent changes to the morphology of the mammary glands. The absence of feeding in a lactating woman leads to irreversible changes that result in involution of the mammary glands and the cessation of lactation. Therefore, understanding the molecular mechanisms of ceasing and restarting lactation would clearly be advantageous to human biology.

Male lactation is not a normal event but does occur in two species of Old World fruit bat [15]. Interestingly, human male lactation has been documented in certain clinical conditions and therefore the biological machinery for lactation exists in males.

Gestation length also exhibits a great deal of variation, not only between species but also within. Although human gestation is 40 weeks or 280 days, between 37 and 42 weeks is considered normal. Pregnancies that continue unabated for longer result in labour being induced to ensure mother and child remain healthy. However, as always, there are exceptions. One human pregnancy has been documented lasting 375 days, approximately 12.5 months. The prenatal doctors described fetal growth as slow but normal, resulting in the birth of a girl weighing a non-exceptional 6 lb 15 oz. The mechanisms that regulate gestation are therefore complex and differ considerably between species depending on the reproductive strategy employed, i.e. the number of offspring and the level of development required when born. For example, for some species such as antelopes, horses and elephants, it is imperative that the newborn is able to be up walking and running within a few hours and therefore gestation is relatively long to enable adequate development. For other species such as mice, cats and dogs, where gestation is relatively short, numerous helpless individuals are born.

Population dynamics

The ultimate goal for an individual, as stated at the beginning of this chapter, is to reproduce, generating offspring capable of passing on the individual's genes. Therefore, of all the offspring produced, for a population to remain stable, each individual has to reproduce a single individual capable of breeding. Consequently, all of the other offspring produced will most likely provide food for other species. Humans in most developed countries are able to make active choices about the number of offspring they produce and have many tools at their disposal to assist with this decision. Contraceptives and awareness ensure that most humans are able to decide when and where to invest their energy to produce the next generation.

Summary

There are many mechanisms employed by different species to enable reproduction to occur successfully. By studying not only human physiology but also that of different species, we enhance our understanding of the mechanisms that regulate physiology and also discover unexpected strategies that, when fully understood, may be able to advance assisted reproductive technology and human health.

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Chapter 2

Sexual development

Andy Greenfield

Introduction

Sex and its anatomical origins have been a source of endless fascination for scientists and philosophers since the time of the ancient Greeks, from chauvinist Aristotelian notions that the semen contributes the 'soul' of the fetus while the female contributes mere matter, through to Galen's contention that the right testis produces seed that generates a boy while that from the left, a girl. Many proposals such as these, based on evidence of varying quality, have been made historically and have not stood the test of time. The advent of improved microscopy, genetics and, latterly, molecular biology has resulted in a contemporary, sophisticated understanding of how male and female newborns appear in approximately equal ratios. This chapter reviews some of our current understanding of mammalian sexual development. It is in no way an exhaustive review, but rather aims to act as a primer for further study of the literature.

Two experimental observations still form the basis of our understanding of how sex is established in humans and other mammals: (i) The pioneering experiments of Alfred Jost established that castrated mammalian embryos develop as females. From this he concluded that male development is induced in the embryo by the activity of sex hormones produced by the fetal testis. This observation is the experimental basis of the much misunderstood remark that female development is a 'default' pathway; (ii) The genetic demonstration that the Y chromosome is a dominant male determinant, in the late 1950s, suggested that, once embryonic chromosomal sex is established at conception, the Y chromosome is necessary and sufficient to cause testis development. XY embryos develop as males because, first, testis differentiation occurs and, second, because the testis produces hormones that masculinize extra-gonadal tissues. If an ovary develops, such as in an XX embryo, or if no gonad is present, the anatomical outcome is female.

It is the intention of this chapter to provide an overview of the sequential events required for normal male and female development. I will then offer a more detailed account of the cell lineages of the developing gonads and how these are established within a bipotential gonadal primordium. Finally, I will examine what is known about the genes/proteins required to orchestrate sexual development.

Development of the reproductive organs: an overview

The mammalian fetus is sexually dimorphic, that is, it comes in two distinct 'flavours': male and female. However, these distinct sexes arise from what are anatomically indistinguishable starting points in the early embryo. For example, human embryos, whether they are XX or XY in chromosome constitution, develop in an identical fashion for the first two months of gestation. Only subsequently do the fates of the primordial reproductive organs diverge. In the mouse, an important model organism for the study of normal and abnormal sexual development and the focus of much of this chapter, the first 11–12 days of development (out of a total of around 19–21 days) appear indistinguishable in XY and XX embryos. In this section we will see how evolution has solved the problem of how to generate sexually dimorphic reproductive organs, with an interesting difference between the gonads and the associated reproductive tracts. The testis and ovary arise from a single bipotential primordium (the genital ridge), while the male and female reproductive tracts develop from distinct primordia (the Wolffian and Müllerian ducts, respectively) that are both present in the early embryo. Excellent reviews

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

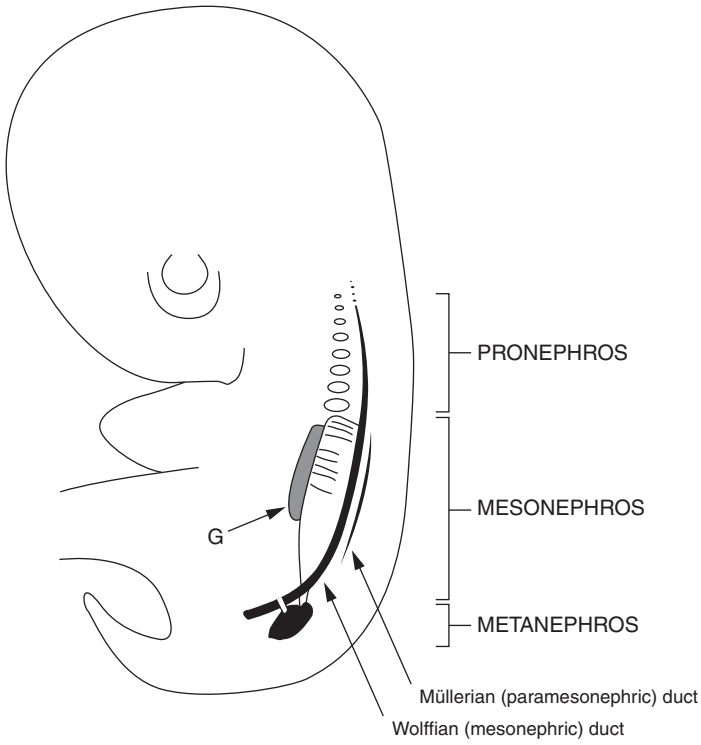

Figure 2.1 The developmental origins of the urogenital system. The three embryonic regions (pronephros, mesonephros, metanephros) are indicated, as are the developing male (Wolffian) and female (Müllerian) reproductive tracts. The gonad (G) forms on the surface of the mesonephros.

of these and other aspects of sexual development can be found in [1], [2] and [3].

The mammalian gonad develops as an integral part of the urogenital system. This is itself derived from intermediate mesoderm that runs along the length of the embryo either side of the midline. Traditionally, the urogenital system is divided into three segments distinguished from anterior to posterior as the pronephros, mesonephros and metanephros (Fig. 2.1), which arise along the Wolffian (nephric) duct. In mammals the pronephros is vestigial and the metanephros gives rise to the permanent kidney. The mesonephros acts as an excretory organ in some species, but it has a much more significant role to play in the development of the embryonic gonad. The gonads are first apparent on the ventromedial surface of the mesonephros at around 10 days *post coitum* (dpc) in the mouse. They are often called genital ridges at this stage and from around 10.5 dpc are composed of somatic cells derived from the mesonephros and primordial germ cells. The latter migrate into the gonad from their site of origin at the base of the allantois. At around 11.5 dpc in the mouse, the gonads of XX and XY embryos are indistinguishable by microscopic investigation of their morphology, although analysis of gene expression at this stage indicates that each organ has already become committed to a distinct fate. The term 'sex determination' refers to this commitment and intensive study over the last 30 years has shed light on its molecular basis. Subsequent to this commitment, the male and female gonads undergo a series of complex molecular and cellular events resulting in the differentiation of the testis or ovary. By 13.5 dpc in the mouse, the testis is clearly distinguishable in the XY embryo on the basis of its larger size, pattern of vascularization and, most notably, the appearance of testis cords. At this stage, the embryonic testis already exhibits the anatomical pattern found in its adult counterpart. In contrast, the ovary is a smaller structure with fewer overt morphological differences when compared to the bipotential gonadal primordium. However, ultrastructural investigations have revealed a distinct pattern of ovarian differentiation. This process of sexually dimorphic differentiation following sex determination was until recently considered to be founded on cell lineage commitment that was essentially irreversible. However, studies of gene function in mutant mice have revealed that ovarian morphology is actively maintained in adult female mice and disruption to genes required for this maintenance can result

in reprogramming of somatic cells and subsequent transdifferentiation of the ovary to a testis [4].

The Wolffian duct first appears in the mouse embryo in transient fashion, associated with the pronephros in the form of short segmental swellings (Fig. 2.1). These subsequently fuse to form a stable, continuous tubular structure that runs the length of the urogenital system, terminating in the cloaca. The ureteric bud, an outgrowth of the Wolffian duct towards its caudal end, interacts with surrounding metanephric mesenchyme to form the metanephros, or permanent kidney. The role of the Wolffian duct in sexual development is to act as the anlage or primordium of the future male reproductive tract structures: the vas deferens, epididymis and seminal vesicle. This developmental programme is dependent on the presence of testosterone produced by the testis.

The Müllerian duct, the primordium of the female reproductive tract, forms from around 12.0 dpc in the mouse from cells at the anterior end of the mesonephros derived from the coelomic epithelium. An epithelial anlage then segregates from the coelomic epithelium and extends caudally through a process involving rapid cell proliferation [5]. The Müllerian duct runs parallel to the Wolffian duct, in a lateral position, for the length of the mesonephros before turning towards the midline, where it fuses with the contralateral duct before reaching the cloaca. In the female (XX) embryo the Müllerian duct differentiates into the oviduct, uterus and upper vagina. In contrast, due to the absence of testosterone, the Wolffian duct regresses. The converse situation occurs in male (XY) embryos: anti-Müllerian hormone (AMH) from the testis causes the Müllerian duct to regress by a process involving apoptosis. The presence of testosterone promotes Wolffian duct differentiation. In this way, the developing gonad controls the fate of the male and female reproductive tract anlagen (Fig. 2.2).

Later in gestation, another testicular hormone, INSL3, results in descent of the testes into an inguinal

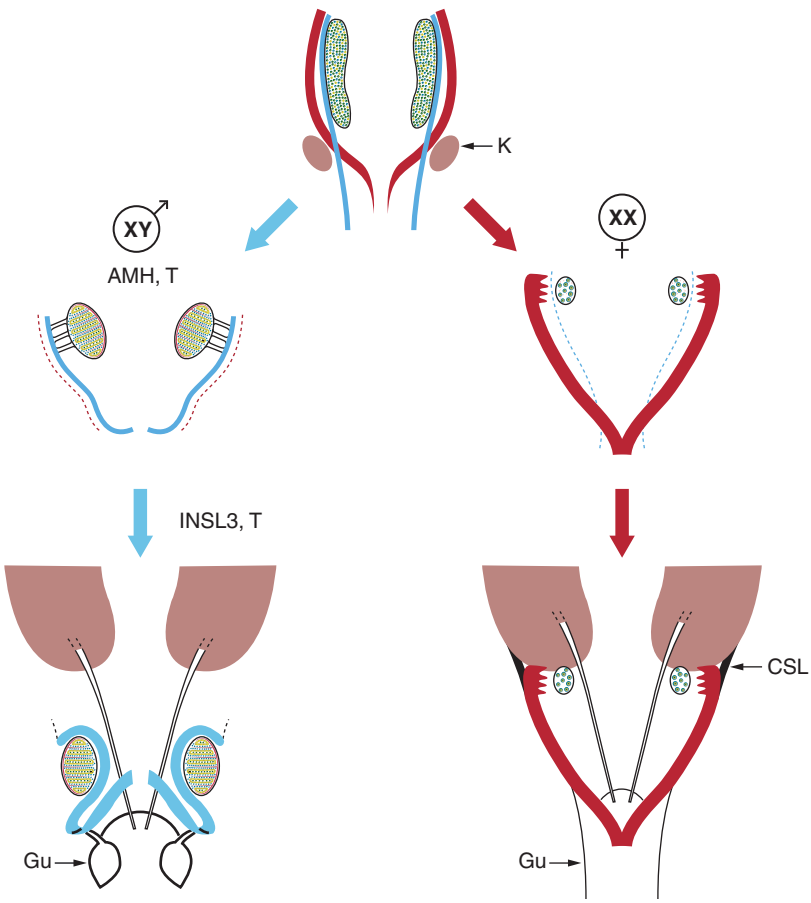

Figure 2.2 Sexually dimorphic development of the male and female reproductive tracts from a morphologically identical ground-state. In XY males, the developing testis produces anti-Müllerian hormone (AMH) and testosterone (T) that result in loss of the Müllerian duct (red) and growth of the Wolffian duct (blue), respectively. INSL3 from Leydig cells also results in growth of the gubernaculum (Gu) and descent of the testis. In contrast, the absence of AMH, T and INSL3 in females results in growth and differentiation of the Müllerian duct and atrophy of the Wolffian duct. The female gubernaculum does not grow and the cranial suspensory ligament (CSL) maintains the ovary in its position close to the kidney (K).

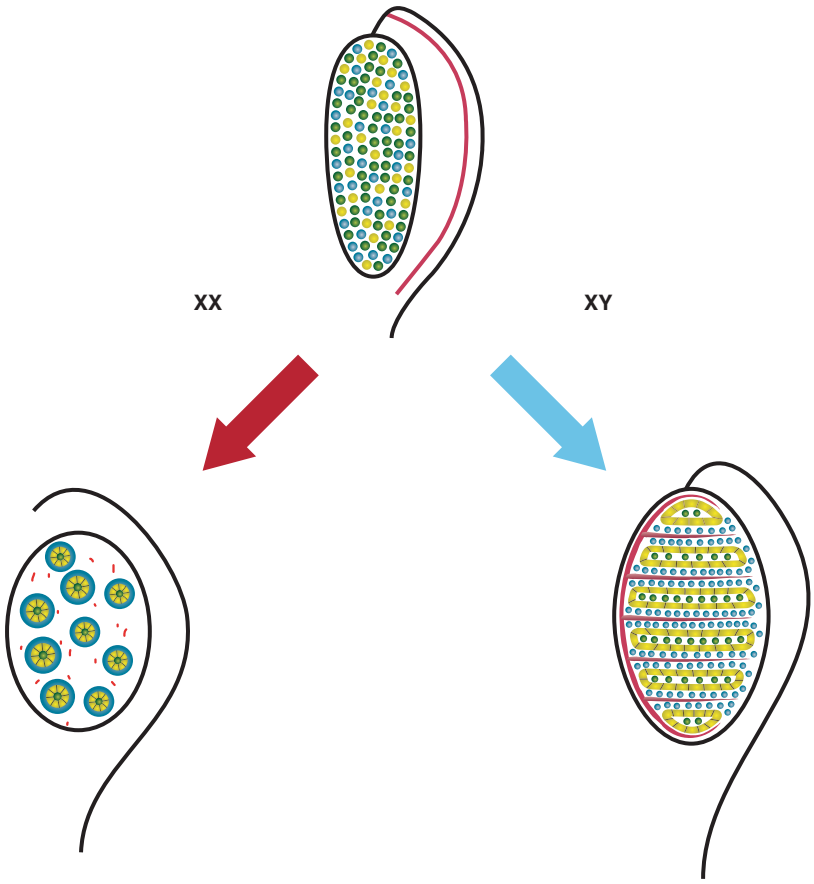

Figure 2.3 Homologous, bipotential cell lineages of the developing ovary and testis. The bipotential XY and XX gonads contain a population of precursor cells (supporting cell (yellow), germ cell (green) and steroidogenic (blue)) that have the capacity to differentiate into testicular or ovarian cell-types. In XY males the supporting cell precursors differentiate into Sertoli cells and these form seminiferous cords that surround germ cells, causing them to enter mitotic arrest. The steroidogenic precursors develop into interstitial Leydig cells. In XX females, in contrast, supporting cell precursors develop into granulosa cells of primordial follicles and steroidogenic precursors from theca cells. Germ cells enter the first stages of meiosis. The prominent coelomic blood vessel of the testis (red) originates from migratory endothelial cells that originate in the mesonephros.

position within the abdomen, and ultimately into the scrotum. INSL3 acts via its receptor, LGR8, which is present in the gubernaculum, or caudal suspensory ligament. In females, the gubernaculum does not increase in size due to the absence of INSL3 and another ligament, the cranial suspensory ligament, maintains the ovary in its pararenal position high in the abdomen (Fig. 2.2).

In male embryos, androgens also act to masculinize other internal and external genitalia, resulting in the appearance of familiar accessory glands, such as the prostate and bulbourethral glands, and the differentiation of the genital tubercle into the penis. Details of these processes have been reviewed elsewhere [3].

The cell biology of the developing gonads

The bipotentiality of the gonadal primordium is evidenced by its capacity for sex reversal, namely, development of ovaries (or ovotestes) in an XY individual and testes in an XX. Gonadal sex reversal is observed in mouse mutants and in humans exhibiting disorders of sexual development (DSD). Moreover, a number of key cell lineages in the testis and ovary are thought to be homologous, i.e. a single bipotential cell lineage gives rise to a testicular cell type or its ovarian counterpart, depending on the genotype of the individual (see overview in Fig. 2.3). In this section we will consider the key gonadal cell lineages in turn.

Supporting cell lineage

Called supporting cells because of their role in the maturation of germ cells, this lineage includes testicular Sertoli cells and ovarian follicle cells. In the male gonad the Sertoli cells are the first cell type known to differentiate and they are thought to act as the organizing centre of the masculinizing signal that drives testis development. The Y-linked testis determining gene, *SRY*, acts in this lineage through its expression in pre-Sertoli cells (between approximately 10.5 and

12.0 dpc in the mouse). Sertoli cells reside within testis cords once these have formed, where they abut the surrounding basement membrane and form close connections with germ cells in the centre of the cord. The origin of Sertoli cells has been a matter of some contention, but at least some are known to arise from proliferative cells in the coelomic epithelium overlying the gonad [6]. Still less is known directly about the origin of ovarian follicle cells; however, based on the thesis of homology, both Sertoli and follicle cells will have the same origin.

Germ cells

Germ cells are not essential for organogenesis of the testis since XY mouse mutants lacking germ cells still develop testes. Once sequestered inside the developing testis cords, XY germ cells enter mitotic arrest and do not resume division until after birth. In XX gonads, germ cells are required for the initial organization of the ovary into follicles and follicular growth. However, they do not appear to be required for maintenance of ovarian somatic cell identity during development [4]. XX germ cells enter meiotic arrest at around 13.5 dpc in the mouse. While this entry into meiosis was once thought to be a cell-autonomous, clocklike phenomenon, it is now known that retinoic acid (RA) acts as a meiosis-inducing factor. The developing testis acts to counter this retinoid signal by the production of an enzyme, CYP26B1, which degrades RA [7]. Sequestration of germ cells within testis cords is also likely to protect XY germ cells from exposure to residual RA.

Steroidogenic cells

Organ culture experiments in mouse reveal that Leydig cell precursors are already present in the bipotential primordium by 11.5 dpc, although their origin is unclear. It has been proposed that they arise from migratory mesonephric cells, cells from the adrenal primordium or perivascular cells. In the female gonad these precursors develop into theca cells. In the male, Leydig cells differentiate due to signals from pre-Sertoli cells and populate the regions between the testis cords, known as the interstitium. They play a key role in masculinizing the embryo by producing the hormones dihydrotestosterone, testosterone and INSL3. It is thought that fetal Leydig cells are replaced after birth by a population of adult Leydig cells.

Endothelial cells

The developing testis is characterized by a prominent blood vessel on its coelomic surface, the coelomic vessel, which is important for the export of androgens. Tributaries of this main vessel are also found running between the testis cords. Organ culture studies in the mouse have revealed the key role of the mesonephros in the formation of testis cords and the coelomic vessel. When cultured in vitro in the absence of the adjacent mesonephros from 11.5 dpc, XY gonads fail to form testis cords. Testis cords form almost normally when the mesonephros is not removed prior to culture. When a fluorescently labelled mesonephros is cultured in vitro adjacent to an XY gonad, the contribution of mesonephric cells to the developing testis is revealed by the subsequent detection of labelled gonadal cells. These migratory cells are endothelial [8]. Such migration does not occur when an XX gonad is co-cultured with a labelled mesonephros, indicating that (i) mesonephric cell migration is male-specific and is based on a chemotactic signal in the gonad produced as a consequence of *SRY* expression; (ii) the mesonephros contributes almost exclusively endothelial cells to the XY gonad; (iii) the endothelial cell lineage plays an instructive role in testis cord formation. Endothelial cells in the developing ovary are found in smaller numbers not regulated by mesonephric cell migration.

Peritubular myoid cells

Peritubular myoid cells (PMC) are an exception to the homology thesis: they are a testis-specific cell type of unknown origin and with no known counterpart in the ovary. This smooth muscle-like cell, which in the adult testis contributes to the movement of sperm along the seminiferous tubules by peristaltic contractions, surrounds the basement membrane of the newly formed testis cord. Studies in vitro suggest that PMC and Sertoli cells interact in order to deposit the basement membrane and provide the cord's structural integrity.

The genetic control of sexual development

Identification of SRY and its role in testis determination

The identification of the Y-linked mammalian testis determining gene, SRY, is perhaps the best example of the impact made by the 'new genetics' in the 1980s and

also the importance of rare cases of human sex reversal in identifying sex-determining genes. In the case of SRY, the individuals that turned out to be most informative exhibited the rare disorder of XX male development. Careful analysis of these cases using new tools in molecular genetics revealed the existence of very small fragments of the Y chromosome in their genomes [9]. These fragments, which had been transferred to the paternally derived X chromosome through an illegitimate recombination event, were postulated to encode the much sought for human testis-determining factor (known as TDF). Since it is a dominant male determinant, a single copy of TDF on an XX genetic background would be sufficient to cause male development, although the presence of two X chromosomes in such males renders them infertile. After one abortive attempt to establish the identity of another gene, *ZFY*, with TDF, the correct gene (SRY) was identified, as evidenced by the male development of XX mouse embryos made transgenic for *Sry* [10]. Indeed, other criteria met by SRY supporting its unparalleled candidacy for TDF, including male-specific expression in the somatic cells of the developing genital ridge, conservation on other mammalian Y chromosomes and loss-of-function mutations associated with XY female development, have been used subsequently to define additional, autosomal testis determining genes, such as *SOX9*. SRY contains a DNA-binding domain, the HMG box, which immediately suggested that it was regulator of gene expression in pre-Sertoli cells [9]. However, due to the frequency of its DNA target sequence in the genome, it took several years to define the key target gene whose transcription was regulated by SRY: *SOX9*.

SOX9: a key vertebrate testis-determining gene

SOX9 was first identified as a gene required for testis development after it was found to be disrupted in patients with campomelic dysplasia (CD) [11]. CD is characterized by skeletal abnormalities, but a number of XY individuals also develop as phenotypic females, suggesting that the gene responsible for CD functions in both chondrogenesis and testis development. Studies of *Sox9* transcription in mouse embryos, using *in situ* hybridization, revealed prominent expression in developing skeletal structures and the testis, but not ovary. Detailed analysis of gonadal expression showed that *Sox9* was expressed at high levels in somatic cells of the early XY gonad and subsequently in Sertoli cells of the testis cords. Thus, the supporting cell lineage exhibited expression of both SRY and *SOX9*, suggesting a possible direct regulatory relationship between the two. Studies of protein expression then revealed that SRY could be detected from about 10.75 dpc in cells in the central region of the XY gonad. By 11.5 dpc, some individual cells contained both SRY and SOX9 and these could be detected at the centre and poles of the gonad. By 12.5 dpc, SOX9-positive cells were detected throughout the gonad, while SRY was absent. Molecular studies of the *SOX9* regulatory sequences that drive gonadal expression then revealed that SRY, and another protein, SF-1, could bind to *SOX9* and upregulate its transcription [12]. These studies defined some of the first molecular and cellular events required for testis development.

SOX9 proved to be a key determinant of testis development in vertebrates. XX embryonic mouse gonads can be driven towards a testicular fate by forced expression of *Sox9*, and organisms as diverse as chicken and fish also showed male-specific expression of cognate genes. *SOX9* encodes another HMG-box transcription factor, and so after its discovery the search then began for those genes regulated by SOX9 that control Sertoli cell differentiation and other male-specific processes, such as mesonephric cell migration and enhanced growth.

Spreading the masculinizing signal: FGF9

The usefulness of gene inactivation studies in the mouse in identifying novel sex-determining genes was revealed when mice lacking fibroblast growth factor 9 (FGF9) were shown to exhibit XY gonadal sex reversal [13]. FGF9 is a secreted signalling molecule that plays a role in other embryonic structures, including the lungs and limbs, in order to control processes such as cell proliferation and differentiation. By establishing a requirement for FGF9 during testis development, a role for cell-cell communication, and thus non-cell autonomous activity, in gonad development was verified at the molecular level. Such cell-cell interactions have long been known to play a role in testis development, ever since the analysis of chimaeric XX-XY mouse embryos, generated by fusion of XY and XX morulae, demonstrated that the presence of at least 20–30% XY cells in the developing gonad could drive XX somatic cells to contribute to a developing testis. Paracrine signals produced by XY somatic cells, presumably pre-Sertoli cells, could recruit XX cells to a male fate. Occasionally, even XX Sertoli cells could be identified in such chimaeras [14].

As in the case of SRY and SOX9, studies of *Fgf9* in mouse gonads reveal a sexually dimorphic pattern of expression. *Fgf9* expression is detected by 11.5 dpc in the XY gonad, but is absent from XX gonads, and exhibits a now familiar centre-to-pole dynamic profile. This suggests that its early expression is regulated by SRY/SOX9. The FGF9 receptor, FGFR2, is expressed in pre-Sertoli cells and in cells of the coelomic epithelium, and ablation of the *Fgfr2* gene also results in male-to-female sex reversal to varying degrees, depending on genetic background [15, 16]. These genetic ablation studies indicate that FGF9/FGFR2 play a role in male-specific proliferation in the coelomic domain of the gonad, in addition to Sertoli cell differentiation. Recent studies that again take advantage of organ culture techniques in the mouse indicate that diffusible FGF9 acts to spread the initial, central masculinizing signal of SRY/SOX9 protein towards the gonadal poles [17]. In these regions FGF9 acts to maintain *Sox9* transcription at high levels and recruit additional cells to the Sertoli cell fate, perhaps by initiating *Sry* expression. The upshot of this dynamic molecular genetic interplay, which involves positive and negative feedback loops, is the rapid establishment of the testiculogenic programme throughout the length of the gonad. Thus, despite the disparate timing of SRY expression in the centre and pole regions of the gonad, there is no appreciable difference in the timing of testis cord formation in different regions of the gonad. Moreover, the rapid spread of the masculinizing signal acts to prevent any female-promoting signals that might persist within the gonadal primordium. In the mouse, the possibility of such ovary-promoting events gaining hold in the developing testis is evidenced by ovotestis formation. In ovotestes, the pole regions of the gonads typically exhibit signs of ovarian differentiation, exemplifying the increased risk that the polar regions incur due to the delay in their receipt of the masculinizing signal initiated by SRY. These observations underline the concept of the gonad as a developing organ with a strongly canalized fate: either testis or ovary. The identity of ovarian-determining genes and the antagonism between the testis- and ovary-determining pathways is the subject of the next section.

Building the ovary

The existence of ovarian determining genes, and their possible role in antagonizing the programme of testis determination, had been predicted on the basis of rare cases of XX male development in the human population [18]. In certain XX males, no SRY sequences exist to explain the female-to-male sex reversal. Some other mutation must be responsible for the phenotype of these individuals: but as any student of evolution knows, gain-of-function mutations are much, much rarer than loss-of-function. The best explanation for SRY-negative XX male development, therefore, is the loss of an ovary-determining gene that also acts to antagonize male development.

In 2006, just such a gene was identified after study of a consanguineous family including several individuals exhibiting palmoplantar hyperkeratosis and XX male development [19]. The gene responsible, R-spondin1 (RSPO1), is an orphan ligand that activates Wnt/β-catenin signalling, a pathway with established roles in development and disease. The discovery of RSPO1 as an anti-testis/pro-ovary gene immediately confirmed the potential role of Wnt/β-catenin signalling in antagonizing testis development. This association had already been made due to earlier studies in the mouse, in which XX mice lacking a component of the Wnt signalling pathway, WNT4, exhibited partial female-to-male sex reversal, including the formation of a coelomic vessel in the developing ovary and ectopic testosterone biosynthesis due to inappropriate migration of endothelial and adrenocortical cells into the developing ovary [20]. Gene expression studies in mice showed that *Wnt4*, like *Rspo1*, is expressed at high levels in the developing ovary but is not detected in the developing testis. Moreover, the partial sex reversal in *Wnt4*-deficient XX gonads is associated with inappropriate expression, at least transiently, of testis-determining genes such as *Fgf9* and *Sox9* [21]. The role of the Wnt/β-catenin pathway in promoting ovary development was subsequently confirmed by genetic ablation of β-catenin in the developing XX gonad, which resulted in partially masculinized gonads with a phenotype strongly reminiscent of those found in XX embryos lacking *Rspo1* and *Wnt4* [22]. Thus, these three genes combine to promote ovary differentiation and (thereby) antagonize the testis-determining pathway. However, female-to-male sex reversal in mice lacking *Rspo1*, *Wnt4* or β-catenin (Ctnnb1) is only partial, suggesting the possible existence of further ovary-determining genes.

Maintaining the ovary: FOXL2

FOXL2, a forkhead transcription factor, was first implicated in ovarian development and function

when heterozygous mutations in the gene were detected in BPES, a syndrome associated with premature ovarian failure. Moreover, once a deletion of FOXL2 was established as the cause of the polled-intersex (PIS) phenotype, an example of XX male development in the goat, it became clear that it might also play a repressive role with respect to testis development. Loss-of-function studies in the mouse verified this predicted role by revealing female infertility in *Foxl2*-deficient XX animals caused by a blockage of follicle development and, crucially, activation of the somatic testis-determining pathway in mutant ovaries after birth. The reactivation of the male pathway suggests a role for FOXL2 in active maintenance of the ovarian state. Indeed, recent studies using conditional gene ablation in the mouse show that FOXL2 is required in the adult ovary to prevent transdifferentiation of granulosa and theca cell lineages into Sertoli-like and Leydig cell-like lineages [4].

As in the case of other genes required for normal ovary development, sex reversal was only partial, suggesting that no single ovarian determination gene exists, in contrast to the testis-determining role of SRY. However, SOX9-positive, testis-like tubules form in newborn gonads from XX mice lacking both FOXL2 and WNT4 [23]. This sex reversal also extends to germ cells, with the formation of spermatogonia. Thus, FOXL2 and WNT4 combine to suppress the somatic and germ cell differentiation common to testicular morphogenesis, and primary sex reversal ensues in their joint absence.

The opposing forces of male and female sex-determining genes

Evidence that antagonism between the testis- and ovary-determining pathways is mutual came from examination of gonad development in XY embryos lacking *Fgf9* [21]. These are characterized by the initial expression of *Sox9* as in wild-type embryos, but a subsequent failure to maintain levels of *Sox9* expression required for Sertoli cell differentiation and testis cord formation. Along with greatly reduced expression of *Fgf9* in embryonic gonads lacking SOX9, these data suggest that a positive feedback loop exists by which *Sox9* and *Fgf9* mutually promote each other's expression. Moreover, the abortive attempt to establish the male pathway in *Fgf9*-deficient gonads is associated with up-regulation of *Wnt4*, indicating that FGF9 is required to down-regulate *Wnt4* expression in developing XY gonads. Thus, the testis- and ovary-determining pathways antagonize each other and loss of one is associated with activation of the other. This scenario is sometimes described as a 'battle of the sexes', although this expression may, to some, have a connotation of drama. So it should be remembered that the vast majority of XY gonads develop as testes, and XX gonads as ovaries. The antagonism revealed by genetic studies reflects an organogenetic programme that is strongly canalized once divergence from a common developmental origin is initiated. Presumably, the bipotentiality of the developing gonad increases the risk that divergent genetic programmes natural to that primordium might attempt to run at the same time with disastrous consequences, and thus an evolved, post-commitment antagonism 'makes sense'. But the battle is really one that was fought over evolutionary timescales, rather than one that flirts with potential disaster in a regular fashion: the outcome, like a battle re-enactment, is only rarely in doubt.

Summary and concluding remarks

Although this has been a far from comprehensive review, as evidenced by the limited bibliography, we have seen the importance of studies of disorders of sexual development in humans and mice in piecing together the molecular genetic and cellular pathways comprising testis and ovary development. Although much remains to be understood, a framework now exists in which new questions can be formulated and addressed (Fig. 2.4). We have focused on the similarities between human and mouse gonad development here, but several differences exist [24]. However, a summary of gonad development can be given that applies to both species: the bipotential gonadal primordium arises in close association with the mesonephros and is populated by precursors of distinct gonadal cell types from a variety of origins, including Sertoli cell precursors from proliferative cells of the coelomic epithelium, endothelial cells from the mesonephros and germ cells from the vicinity of the allantois. In XY gonads, somatic cells in the centre of the gonad express SRY, a process dependent on transcription factor activity regulated by insulin-related growth factors and mitogen-activated protein kinase (MAPK) signalling [25]. SRY protein up-regulates transcription of SOX9, which itself results in FGF9 expression. The masculinizing signal of SRY/SOX9/FGF9 rapidly radiates along the gonad, promoting Sertoli cell differentiation, cell proliferation in the coelomic region, mesonephric cell migration and testis cord and

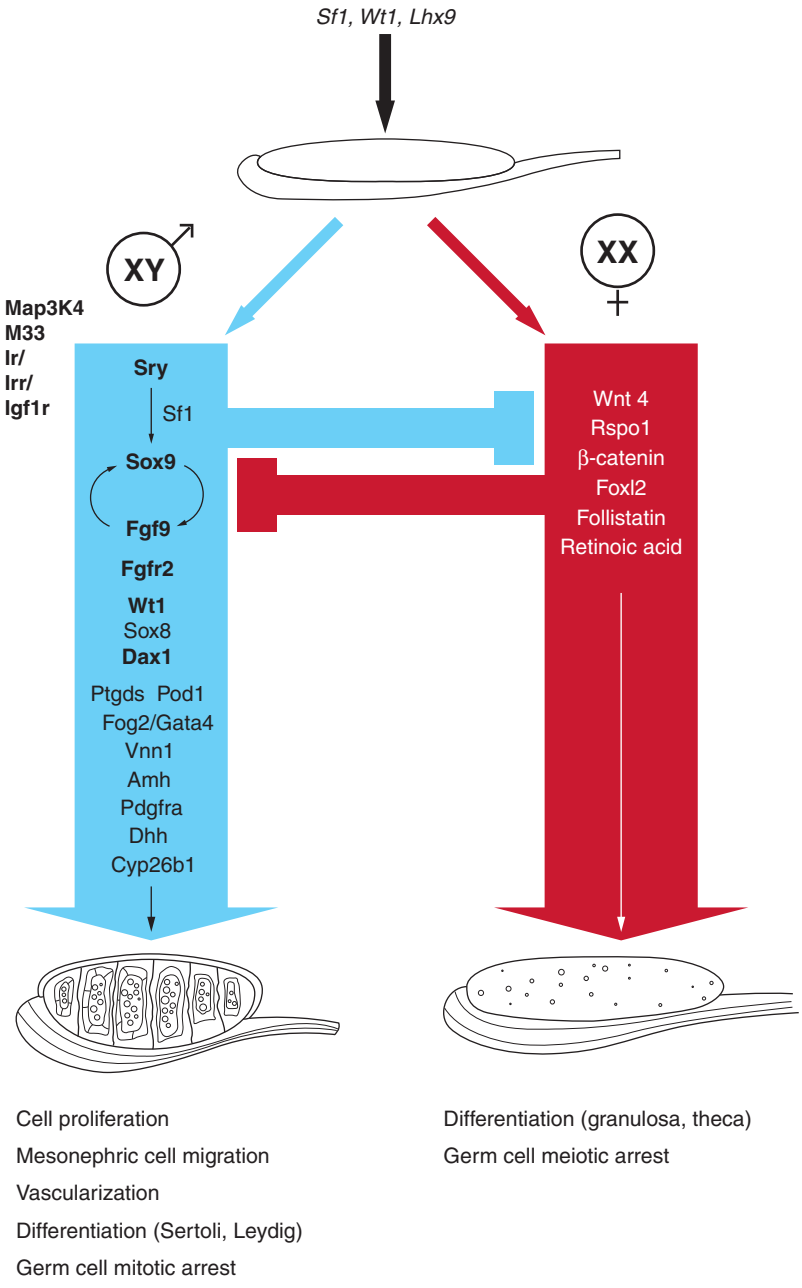

Figure 2.4 The mutual antagonism between the testis- and ovary-determining genetic pathways. In XY males, SOX9/FGF9 act to repress the activity and expression of ovarian promoting gene products such as WNT4, β-catenin and FOXL2. The converse occurs in the XX gonad. This mutual antagonism promotes sex-specific cellular processes and results in the strongly canalized fate of the gonads in XY and XX embryos. Genes contributing to the male and female pathways are shown. *Sf1*, *Wt1* and *Lhx9* are also required for formation of the gonad primordium.

coelomic vessel formation. At the same time, any residual ovarian-determining genes are repressed, underlying the canalization of gonad development. Signals from Sertoli cells result in Leydig cell differentiation in the interstitium and mitotic arrest of germ cells in the testis cords. At this stage, the morphological structure of the testis is established and differs little from the pattern found in the adult testis. Hormones from the Leydig cells, including testosterone and INSL3, masculinize the reproductive tracts and external genitalia and result in testicular descent. In contrast, in the absence of SRY, supporting cell precursors establish an ovarian-promoting milieu in the gonad, driven by *FOXL2*, *WNT4/CTNNB1* and *RSPO1*, and other genes not discussed in this chapter. Granulosa and theca cells differentiate and germ cells enter meiotic arrest. Testis-determining genes such as *FGF9* and *SOX9* are repressed, as is coelomic zone growth and

mesonephric cell migration. Germ cells and continued expression of ovarian-determining genes help to maintain ovarian identity throughout adult life.

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Chapter 3

The male reproductive tract and spermatogenesis

Joaquin Gadea, John Parrington, Junaid Kashir and Kevin Coward

Objectives

The purpose of the male reproductive system is to (i) produce, maintain and transport sperm and seminal plasma; (ii) discharge sperm within the female reproductive tract; and (iii) produce and secrete androgens for maintaining male reproductive capacity. The objective of this chapter is to briefly review the key components of the male reproductive system and explore their basic structure and functional role. Hormonal regulation and the process of spermatogenesis will also be examined.

Structure and function of the male reproductive system

Reproduction is the process by which organisms create offspring. While both the female and male reproductive systems are involved in producing, nourishing and transporting either the egg or sperm, these systems are very different in shape and structure. The male reproductive organs include the testis, epididymis, vas deferens, accessory glands such as the seminal vesicles, prostate and bulbourethral glands, and the copulatory organ, the penis.

Testes

The testes are the organs that produce sperm, the mature male gametes (Fig. 3.1). The testes also serve important endocrine functions and represent the source of male sex hormones (androgens), the most abundant of which is testosterone. Each testis descends from a retroperitoneal position through the inguinal canal to reach the scrotum during the eighth month of fetal development. Anatomically, the testes are ovoid glands that are suspended in the scrotum. The blood vessels and nerves to the testis stem from within the abdomen in a multilayered structure called the spermatic cord. Each testis is surrounded by a capsule, the tunica albuginea, which is externally covered by a serosa. From the tunica albuginea, fibrous septa project deep into the testis and converge to form the mediastinum. The septa divide the parenchyma of the testis into multiple testicular lobes, each of which contains convoluted seminiferous tubules. The interstitial tissue between the convoluted tubules is continuous with a layer of loose vascular connective tissue, the tunica vasculosa, which is found beneath the tunica albuginea.

Functionally, the testis consists of two compartments: the seminiferous tubules and the intertubular tissue, which forms the interstitium. Seminiferous tubules comprise 95% of testicular volume and are lined by layers of germ cells in various stages of development (spermatogonia, spermatocytes, spermatids, sperm) and supporting Sertoli cells, which provide mechanical and nutritional support for spermatogenic cells. Sertoli cells also secrete inhibin which provides negative feedback on FSH secretion from the pituitary. Spermatogenesis, the process by which male spermatogonia develop into mature sperm, occurs within the seminiferous tubules. Each tubule continues near the mediastinum into a straight tubule, the tubulus rectus. This leads into the rete testis, a labyrinth of cavities in the mediastinum, to connect finally with the epididymis.

In contrast, the interstitium consists of loose connective tissue, blood and lymphatic vessels and various cell types, including Leydig cells, fibroblasts, macrophages and leucocytes. Leydig cells are polygonal in shape and are the major cell type within the

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interstitium where they are often found adjacent to blood vessels and the seminiferous tubules. Leydig cells are the predominant source of the male sex steroid hormone testosterone.

Maintenance of testicular temperature at 2–7°C below body temperature is essential for spermatogenesis to occur efficiently. Several studies have shown that an increase in testicular temperature is associated with morphological abnormalities in sperm and alterations in chromatin structure that may lead to certain forms of infertility [1]. There are a number of anatomical features that favour testicular thermoregulation, such as the presence of a thin scrotal skin with abundant sweat glands and lack of fat, distinct smooth and skeletal muscles responsible for the movement of the scrotum and vascular changes in the morphology of the testicular artery and veins. The contraction of smooth muscle fibres within the subcutaneous tissue of the scrotum and of the cremaster skeletal muscle is an efficient mechanism of thermoregulation. The testes are closer to the abdomen when the ambient temperature is cold and further away when it is hot. In addition, as the testicular artery approaches the testicle, the artery convolutes and is closely surrounded by a plexus of the testicular vein, the pampiniform plexus. This vascular structure generates a very effective counter-current heat exchange mechanism.

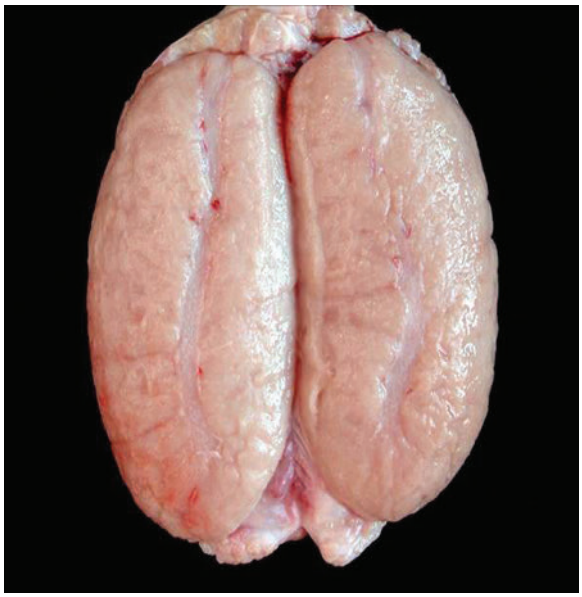

Figure 3.1 Longitudinal section of pig testicles. 1. Parenchyma, 2. Mediastinum (rete testis), 3. Head of the epididymis, 4. Tail of the epididymis (Courtesy of Veterinary Anatomy & Embryology, University of Murcia, Spain).

Epididymis

The epididymis is a coiled segment of the spermatic ducts that serves to store, mature and transport sperm between the testis and the deferen duct (Fig. 3.2). The epididymis can be divided into caput (head), corpus (body) and cauda (tail). A number of efferent tubules from the testis enter the head of the epididymis and join together to form the epididymal duct. This is a very thin and largely convoluted tubule lined by a columnar epithelium containing cilia and microvilli. Sperm take approximately 2 weeks to pass

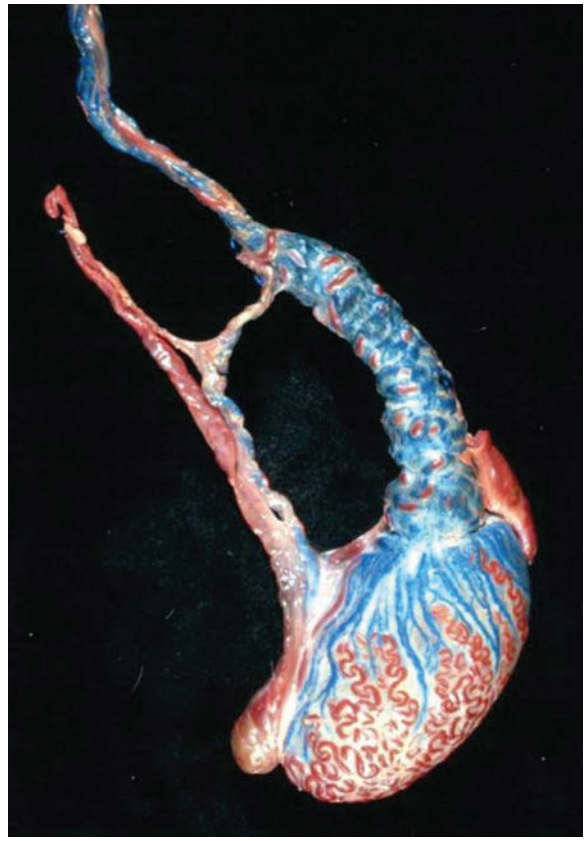

Figure 3.2 Foal testicle injected with coloured latex (red for arteries and blue for veins), medial view. 1. Tunica albuginea, 2. Head of the epididymis, 3. Tail of the epididymis, 4. Proper ligament of the testis, 5. Deferent duct, 6. Testicular artery, 7. Testicular veins over the albuginea, 8. Testicular vein in the spermatic cord (pampiniform plexus) (Courtesy of Veterinary Anatomy & Embryology, University of Murcia, Spain).

through the epididymis. Sperm are stored within the epididymal duct, which also serves to absorb testicular fluid.

As sperm are transported through the epididymis, they undergo important morpho-functional changes. Besides acquiring motility, the midpiece and acrosome are stabilized. The main changes that occur during epididymal transit involve modifications to chromatin within the sperm nucleus, migration of the cytoplasmatic droplet from the neck to a region near the annulus and an alteration in the size of the acrosome. Although the sperm that leave the testicle are fully formed, they are immotile and immature. In the head of the epididymis, fluids from the rete testis are absorbed and replaced by secretions from the epididymal epithelium. As sperm are transported from the head to the tail of the epididymis, changes in the proportions of different proteins in the epididymal fluid occur [2]. These changes lead to modifications in the surface of the sperm plasma membrane such that when the sperm arrive in the tail of the epididymis, they are fully mature. They are then stored in a quiescent state until ejaculation. Membrane alterations may result from the incorporation of proteins, sugars and lipids of epididymal origin, into the sperm membrane. Epididymal sperm also acquire the ability to recognize, bind to and fuse with eggs during epididymal transit.

Vas deferens

The deferent duct, or vas deferens, connects the epididymis to the urethra. The mucosa of the vas deferens is lined by a pseudostratified columnar epithelium, and in a manner similar to the epididymis, its cells have long stereocilia. The muscular layer of this duct is very well developed and consists of a thick circular layer of smooth muscle between thinner inner and outer longitudinal layers. The muscularis is the structure that makes the deferent duct palpable in the spermatic cord. During ejaculation, the smooth muscle of the deferent duct contracts reflexively (peristalsis), thus propelling the sperm and fluids forward and transferring them into the urethra.

Urethra

The urethra extends from the bladder to the tip of the penis and constitutes a common passageway for semen and urine. The two major sections of the urethra, the pelvic part and the penile part, are differentiated. The pelvic part is first surrounded by the prostate gland and then by the striated urethral muscle. In addition to the prostate secretion, at the time of ejaculation, the semen also receives the content of the vesicular and bulbourethral glands. The penile part begins where the urethra enters the bulb of the penis at the level of the pelvic outlet. As it is surrounded by spongy tissue, the penile part is also named the spongy urethra.

Seminal vesicles

Seminal vesicles are lobe-type paired glands located next to the end of the deferent duct. Secretion contributes a gel-fraction to the semen, which constitutes the main (50–70%) and final fraction of the ejaculate. This organ provides proteins, enzymes, fructose, mucus, vitamin C, flavins, phosphorylcholine and prostaglandins to nourish and stabilize the sperm [3]. High fructose concentrations provide nutrient energy for the sperm. Secretions from the seminal vesicles appear during subsequent fractions of ejaculation to produce semen, a liquid that coagulates after coming into contact with the seminal vesicular secretion. The major component of this coagulum is semenogelin I, a 52-kDa protein expressed exclusively in the seminal vesicles.

Prostate gland

The prostate is the largest accessory sex gland in men and is a muscular single gland that surrounds the first inch of the urethra as it emerges from the bladder. Prostate secretions enter the urethra by means of multiple prostatic ducts. The smooth muscle of the prostate gland contracts during ejaculation to contribute to the expulsion of semen from the urethra. While the prostate gland is encapsulated by a fibroelastic tissue layer, the prostate capsule gives rise to septa which extend inward and subdivide the prostate into five lobes: anterior, posterior, medial and two laterals. Within these lobes are the tubuloalveolar or saecular glands, excretory ducts and dense stroma [4].

The prostate produces, stores and secretes a clear, slightly acidic (pH 6.6) fluid comprising up to one-third of the semen volume. This secretion is rich in components such as calcium, zinc, citric acid and acid phosphatase. Phosphatase hydrolyzes phosphorylcholine to choline which is used as a nutrient by the sperm. This secretion additionally contains seminal plasmin, an antimicrobial protein that combats urinary tract infections and prostate-specific antigen (PSA), a protease whose function is to break down the high

molecular weight protein of the seminal coagulum and to help semen liquefy following ejaculation.

Prostatic function is regulated by hormones. The presence of testosterone is essential for maintenance of the structural and functional integrity of the prostate gland. It is common for this gland to increase in size with ageing, and this can lead to microturation problems or even malignant hyperplasia.

Bulbourethral glands

The bulbourethral glands, which are also known as the Cowper's glands, are located distally to the prostate. Each gland has a short duct which empties into the spongy urethra as it enters the root of the penis. Their secretory product is a clear, viscous mucin. As a component of the seminal fluid, this mucin protects the urethra and serves as a lubricant during sexual intercourse. The secretion of gelatinous seminal fluid helps to lubricate the urethra for sperm to pass through, and to help flush out any residual urine or foreign matter. The alkalinity of seminal fluid helps to neutralize the acidic vaginal pH and permits sperm mobility in what might otherwise be an unfavourable environment.

Penis

The penis is the main external genital organ and is divided into three portions, which in a proximal to distal order are named the root, body and free portion, or glans. Structurally the penis is built of three erectile components, two corpa *cavernosum* and one corpus *spongiosus*. The root consists of two crura of cavernous tissue which attach to the sciatic arch, and a central bulb of spongy tissue which surrounds the urethral duct as it comes out of the pelvic cavity. In the body of the penis, the two crura of cavernous tissue fuse and the spongy urethra runs ventrally throughout the urethral groove. In the free portion, which is covered with a fold of skin called the prepuce, the spongy tissue expands so as to form the glans.

Erectile tissue consists of a framework of smooth muscle and connective tissue that contains blood sinuses, which are large, irregular vascular channels. This cavernous tissue is the major erectile component in the body of the penis, as is the spongy tissue in the glans. As the pelvic urethra leaves the pelvic cavity and enters the bulb of the penis, it becomes surrounded by spongy tissue. The spongy or penile urethra ends in an external opening located at the tip of the gland. The male urethra is a passage for both urine and semen. The reproductive function of the penis is to be inserted into a woman's vagina and deliver semen by ejaculation, a response evoked by a complex series of reflexes and the physiological phases of this response have been defined as erection, emission and ejaculation. The ejaculatory response is under the control of the sympathetic nervous system.

Erection is induced by tactile stimulation of the genital region or from visual or emotive stimuli that can stimulate descending parasympathetic pathways from the brain. This type of stimulation induces dilatation of arterioles in the penis (via the helicin arteries) and the venous sinuses. Then, both the spongy and cavernous tissues become engorged with blood. As these erectile bodies are surrounded by a strong fibrous coat, the penis becomes rigid, elongated and increases in girth. Contraction of ischiocavernosus and bulbospongiosus muscles over the root of the penis compresses the venous outflow against the sciatic arch without compromising the arterial supply. At the same time, parasympathetic nerves stimulate the bulbourethral glands to produce a mucoid-like substance to aid lubrication. *Emission* involves contractions of the smooth muscle in the walls of the deferent duct that push sperm into the proximal part of the urethra. At the same time, the seminal vesicles and prostate gland contract and seminal fluid is released into the urethra. At *ejaculation*, the semen is expelled from the posterior urethra by contractions of the bulbocavernous and urethral muscles. Passage of semen from the upper part of the urethra and back into the bladder is normally prevented by sympathetic contraction of the urethral sphincter.

Male reproductive tract development and differentiation

The sex of an embryo is determined at fertilization by the introduction of an X or Y chromosome from the spermatozoon into the fertilized egg. In this way, future males (46XY) and females (46XX) are defined by the presence or absence of a Y chromosome. However the gonads, in the early stages of development, are of an indifferent type and can potentially develop into either testis or ovaries [5].

The primordial germ cells, which are to become eggs and sperm, develop in another part of the embryo entirely from the gonads. At the third week, they then migrate through the tissue of the embryo to the gonad. The germ cells remain in this quiescent state until after birth when they resume proliferation, and some

migrate to the seminiferous tubules of the testis. By the time they arrive, the gonad has prepared itself by becoming male or female. In the male, changes are under the influence of the Y chromosome. The Y functional chromosome has an SRY gene that stimulates an autosomal chromosome to produce H-Y antigen that stimulates the medulla of the undifferentiated gonad to develop into the testes. In the testes, Leydig cells start to produce the hormone testosterone [6].

As the testes develop, their hormones elicit the development of the male secondary sex characteristics or male phenotype. Testosterone influences duct development. In the presence of testosterone, the mesonephric or Wolff duct develops to become the vas deferens and associated structures. Without androgens, the mesonephric duct atrophies and the paramesonephric, or Muller duct, becomes the oviduct and most of the uterus. Muller inhibiting substance is formed by the Sertoli cells of the testes.

In the male, the indifferent gonad responds to the effects of the Y chromosome by developing testicular cords which become horseshoe shaped and enclosed within the thickened tunica albugina of the gonad. The free ends of the horseshoes are in contact with the redundant mesonephric duct. Meanwhile, the mesonephric duct continues to develop and forms the epididymis, the vas deferens and the seminal vesicles.

Like the gonads, the structures that develop into the external genitalia are initially identical in males and females. They develop from the same anlagen: the genital or labioscrotal swelling; the genital or urethral folds; the genital tubercle and the urogenital sinus. The development of the external male phenotype requires the actions of testosterone. In a male fetus, the genital swellings migrate and become the scrotum; the urogenital folds enlarge and enclose the penile urethra and corpus spongiosa; the genital tubercle becomes the glans penis; and the urogenital sinus forms the prostate gland. It is not until the last two-thirds of pregnancy that growth of the male fetal external genitalia takes place and descent of the testes into the scrotal sac is complete. During this period, the ducts are rearranged to pass from the scrotum back into the abdominal wall, through the inguinal canal, to unite with the urethra, the terminal duct of the excretory system (refer to Chapter 2).

Spermatogenesis

Spermatogenesis is a complex biological process of cellular transformation that produces male haploid germ cells from diploid spermatogonial stem cells. In humans, the entire spermatogenic process is very long and lasts more than 70 days. This complex process is initiated in the male testis at the beginning of puberty, since germ cell proliferation and survival depends upon gonadotrophin-dependent mechanisms [7, 8].

Germ cells multiply first by repeated mitotic divisions and then by meiosis, which involves the duplication of chromosomes, genetic recombination and then reduction of chromosomes through two cell divisions to produce spherical haploid spermatids. The transformation of spherical, haploid spermatids into elongate, highly condensed and mature sperm that are released into the seminiferous tubule lumen is called spermiogenesis (Fig. 3.3).

The seminiferous epithelium consists of germ cells that form numerous concentric layers that differentiate into mature sperm as they migrate towards the

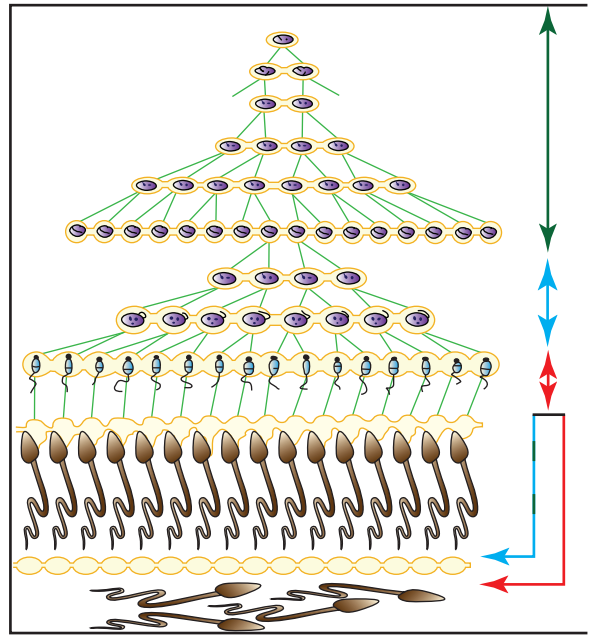

Figure 3.3 Schematic representation of human spermatogenesis. During the ~74 days needed to complete spermatogenesis in humans, the pachytene stage of prophase I takes approximately 14 days, while the remainder of meiosis I and all of meiosis II require < 3 days. Proliferative and meiotic phases are shown by the green line and are under predominantly transcriptional control. Early haploid stages are shown by the blue line and are also under transcriptional control. The red line represents nuclear shutdown in response to post-meiotic translation of sperm RNAs, as described by Miller and Ostermeier [12]. The ultimate fate of these RNA transcripts, and other pre- and early-meiotic RNAs, is thought to be residual bodies (blue and green line). Figure modified and reproduced, with permission, from Miller and Ostermeier [12].

lumen and also the presence of Sertoli cells. These are support cells for the developing gametes, the latter being embedded in the Sertoli cell cytoplasm. Development of the germ cells begins with the spermatogonia at the periphery of the seminal canal and advances across the lumen via primary and secondary spermatocytes, spermatids and finally resulting in mature sperm cells. The maintenance of a normal architecture of the seminiferous tubuli is achieved by a dynamic balance of germ cellular regeneration and elimination by apoptosis.

Sertoli cells are irregular columns in shape in a simple columnar epithelium and they extend from the basement membrane to the luminal surface of the seminiferous epithelium. The nuclei of Sertoli cells usually can be readily recognized by their prominent nucleoli. The Sertoli cell has several important roles in spermatogenesis, including the support and nutrition of the developing germ cells; compartmentalization of the seminiferous tubule by tight junctions, which provides a protected environment for the developing germ cells; controlled release of mature spermatids into the tubular lumen; secretion of fluid, proteins and several growth factors; and phagocytosis of the degenerating germ cells and the excess cytoplasm that remains following sperm formation.

Spermatogenesis can be divided into three phases: spermatogonial (mitosis), spermatocyte (meiosis) and spermatid phases (spermiogenesis).

Mitosis phase

Spermatogonial stem cells reside on the basement of the tubule and divide by mitosis to form spermatogonia. Mitosis is the process of cell duplication – two daughter cells are formed with exactly the same DNA and chromosomal content of the original diploid (2N) mother cell. The spermatogonia are diploid germ cells (2n) and according to the presence and distribution of heterochromatin, spermatogonia are classified as A or B subtypes. In humans, about four generations of spermatogonia are necessary to form preleptotene spermatocytes from one spermatogonial stem cell.

Males have an almost unlimited capacity to produce germ cells; this is accomplished by replenishment of A-spermatogonia early in mitosis, however, the mechanics of renewal are not fully understood.

Meiosis phase

In this phase, the diploid cells created in the spermatogonial phase give rise to haploid cells. B-spermatogonia divide by mitosis forming two preleptotene spermatocytes, which represent the beginning of meiotic prophase. The diploid number of primary spermatocytes is halved during meiosis. A primary spermatocyte is transformed into two secondary spermatocytes (2n) during meiosis I and later these cells form haploid (1n) round spermatids during meiosis II.

Spermiogenesis phase

Spermiogenesis is a process involving the maturation and differentiation of the spherical, haploid spermatids into elongate, highly condensed and mature sperm with potential for movement. During spermiogenesis, male gamete nuclear proteins are altered in order to increase their state of nuclear condensation. There is also a change in cellular size and shape, from a round cell to a flagellated cell, with the formation of the acrosomal cap, derived from the Golgi apparatus and the development of a tail. The localization of the centrioles changes during spermiogenesis, and they migrate to a post-nuclear region upon the completion of meiosis. The distal centriole provides a template for accretion of cytoskeletal elements comprising the contractile function of the tail, and the mitochondria become concentrated into the sheath of the middle piece to generate the energy necessary for the tail movement. Mature sperm are finally liberated by a gradual release from their attachment to the Sertoli cells into the tubule lumen.

Germ cells within each layer of the seminiferous epithelium change in synchrony with the other layers over time, producing a sequence of stages that are clearly evident when serial cross-sections of a seminiferous tubule are examined. A spermatogenic cycle is defined as the time it takes for the reappearance of the same stage within a given segment of the tubule and the distance between two tubules at the same stage, a phenomenon known as the spermatogenic wave.

In humans, spermatogonial development, meiosis and spermiation are the three main processes that are regulated by gonadotrophins. The highly coordinated nature of spermatogenesis requires intimate functional and junctional communications between Sertoli and germ cells. Endocrine factors exert their biological effects on spermatogenesis via receptors located in or on the plasma membrane of Sertoli cells.

Sperm production in adulthood is limited by two factors: the efficiency and normality of the process of

spermatogenesis, and the number of Sertoli cells. Sertoli cells control the process of spermatogenesis, but each Sertoli cell can only support a finite number of germ cells through their development into sperm.

Spermatogonial stem cells (SSCs) can self-renew and generate a large number of differentiated germ cells. A fine balance between SSC renewal, apoptosis events and differentiation in the adult testis is essential in order to maintain normal spermatogenesis and fertility. These processes are regulated by intrinsic gene expression in the stem cells and extrinsic signals. Interactions with the Sertoli cell are crucial for maintaining stem cell character. Several molecules produced by Sertoli cells can regulate the capacity of this niche to support SSC maintenance and differentiation.

Control of differentiation in spermatogenesis is mediated by the successive activation and/or repression of thousands of genes and proteins, including numerous testis-specific isoforms. Advances in molecular biology and genomics have greatly improved our knowledge of spermatogenesis by identifying numerous genes essential for the development of functional male gametes. Recent studies have discovered many transcriptional and post-transcriptional regulatory proteins that regulate spermatogenesis and sperm maturation, which include transcription factors, chromatin-associated factors and RNA-binding proteins [9, 10]. A number of transcription factors have been reported to play a role in germ cells, Sertoli cells and the epididymis. Those expressed in germ cells are more likely to regulate specific phases of germ-cell progression, while Sertoli cell-expressed transcription factors exhibit roles that are often required through all stages of gametogenesis in seminiferous tubules.

During spermatogenesis, spermatid nuclei become transcriptionally inactive as a result of replacement of histones with highly basic protamines, extensive nuclear remodelling and compaction of the chromatin [11]. Chromatin regulators control key epigenetic events, such as histone modifications. Many events, including chromatin condensation, reorganization of the spermatid nucleus and formation of the acrosome and flagellum, require the synthesis of a number of spermatid-specific histone-like proteins. Post-transcriptional events are controlled by RNA-binding proteins. Gonadotrophin-regulated testicular RNA helicase (GRTH) is one of these translation-promoting proteins and increases the survival of both spermatocytes and round spermatids.

Hormonal regulation

The continual production of sperm by sexually mature males requires an efficient and highly regulated process in the seminiferous tubules. Both testicular functions (production of sperm and the synthesis of testosterone) are regulated by hormones in a classic endocrine feedback loop involving follicle stimulating hormone (FSH) and luteinizing hormone (LH) as the key hormonal signals [8].

Pulsatile hypothalamic release of GnRH stimulates the secretion of FSH and LH by the anterior hypophysis. GnRH is a decapeptide that is synthesized in the hypothalamus and carried by the hypothalamico–hypophysial portal system to the anterior pituitary. Binding of GnRH to receptors on pituitary gonadotropes leads to the release of both FSH and LH.

These hormones are heterodimeric glycoproteins (with two subunits: α and β). α subunits are identical for LH and FSH and contain 92 amino acids, while β subunits are different (LH has a β subunit of 121 amino acids while FSH has 118 amino acids), thus conferring capacity for interaction with specific receptors.

Both hormones act at the testicular level. LH stimulates testosterone production by the Leydig interstitial cells, and FSH stimulates Sertoli cells to support spermatogenesis. Indeed, FSH receptors are expressed only in Sertoli cells, and Leydig cells are the only binding sites for LH in the testis. Nevertheless, LH also promotes spermatogenesis indirectly by increasing intratesticular testosterone.

FSH and LH both act through classic protein hormone receptor mechanisms, via a protein associated trans-membrane receptor. Leydig cells adopt the structure of a steroid secreting cell in that they possess abundant smooth endoplasmic reticulum and numerous mitochondria. They are able to synthesize cholesterol from acetate or to take up this substrate for steroidogenesis from lipoproteins present in circulation. Transport of cholesterol is acutely regulated by LH and occurs in two steps: mobilization of cholesterol from cellular stores such as lipid droplets or the plasma membrane to the outer mitochondrial membrane and then the transfer of cholesterol from the outer to the inner mitochondria membranes. LH

directly stimulates the synthesis of a steroidogenic acute regulatory (StAR) protein, which accelerates the transfer of cholesterol from the outer to the inner mitochondrial membrane. In testes, testosterone binds to androgen-binding protein secreted by Sertoli cells. In addition to testosterone and other steroid end products, Leydig cells secrete proteins that may affect spermatogenesis either directly or indirectly. Among these proteins, insulin-like protein 3 (INSL3) has received particular attention because it has been shown to stimulate testicular descent during development. Leydig cells also produce and secrete oxytocin, under the influence of LH, which also stimulates testosterone production. Finally, the intratesticular levels of testosterone are very high compared to blood.

An initial active stage of Leydig cells occurs during the embryonic development of the testis. Later in juvenile life, due to the influence of the LH secreted by the anterior hypophysis, Leydig cells enter into a second stage of activity. Together with the hormones secreted by the adrenal cortex, testosterone initiates puberty and thus the maturation of the sperm cells. Testosterone is also carried away via blood and lymph fluid. Testosterone has effects on all tissues, especially on the brain during development as well as on the sexual organs.

On the other hand, FSH stimulation of Sertoli cells is known to activate different signalling pathways that eventually activate cAMP responsive element (CRE)-binding protein (CREB), leading to the transcription of downstream target genes of spermatogenesis. Sertoli cells provide critical factors necessary for the successful progression of spermatogonia into sperm. Glycoproteins secreted by Sertoli cells important for spermatogenesis can be divided into three categories: (a) those that facilitate the transport of ions and hormones or provide bioprotective functions, such as androgen-binding protein (ABP), transferrin and ceruloplasmin; (b) proteases and protease inhibitors, which have a role in tissue remodelling processes; and (c) structural components of the basement membrane between the Sertoli cells and the peritubular cells.

Testosterone exerts its biological effects on spermatogenesis via androgen receptors that are localized in the Sertoli cells. A requirement for androgen receptor activity in Sertoli cells exists at progression through meiosis I; transition from the round to elongating spermatid; and during spermiogenesis. Androgen action is evidently not needed for passage of the germ cell through meiosis.

Testicular hormones decrease gonadotropin release both by decreasing GnRH production and by decreasing the sensitivity of the pituitary to GnRH stimulation. Also, serum testosterone and inhibin B downregulate LH and FSH secretion via negative feedback loops. Inhibin B is produced in the Sertoli cells under the stimulatory influence of FSH and acts to inhibit FSH secretion from the pituitary.

Summary

The reproductive system is highly complex and specialized to suit a specific functional role. The purpose of the male reproductive system is to produce and release semen into the reproductive system of the female. Spermatogenesis is a complex biological process of cellular transformation that produces male haploid germ cells from diploid spermatogonial stem cells. The continual production of sperm by sexually mature males requires an efficient and highly regulated process in the seminiferous tubules. Both testicular functions (production of sperm and the synthesis of testosterone) are regulated by hormones in a classic endocrine feedback loop. Alterations in the hormone control and defects in spermatogenesis are thought to be a major cause of idiopathic infertility. Emerging knowledge concerning the transcription and post-transcriptional regulation of spermatogenesis will open new opportunities for the diagnosis and treatment of this kind of infertility.

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Chapter 4

Female reproductive tract and oocyte development

Suzannah A. Williams

Introduction

The human female reproductive tract has a number of critical functions. It is not only required to produce functional gametes as in the male, but also to provide an appropriate environment for fertilization of the ovulated egg to occur, to support early embryo development, to ensure the uterus is primed for implantation and to nourish and nurture the development of the growing fetus until birth. To achieve these multiple functions, multiple organs and tissues are required with complex regulatory mechanisms. It is the intention of this chapter to first describe these organs and then discuss their function as it pertains to the generation of a child.

The female reproductive tract consists of the ovary where oocytes develop and from where mature eggs are ovulated. Eggs are ovulated into the fallopian tubes, also known as oviducts, and it is within these tubes that fertilization occurs. The fertilized oocyte, known as a zygote, begins a course of cell division which marks the beginning of embryonic development. The conceptus continues to develop (discussed in Chapter 12) as it passes through the oviduct and into the uterus, where the mature blastocyst hatches, implants and continues to develop into a fully formed fetus. At birth, the fetus is expelled from the uterus and passes through the cervix, down the vagina and is born (Fig. 4.1a). The first part of this chapter describes each part of the female reproductive tract while the second part returns to the topic of female gamete generation. Although the focus is primarily upon human reproduction, studies in other species will also be included where they lend insight into function. Indeed, a number of animal studies are directly relevant as such investigations routinely form the basis for human research owing to ethical reasons and the scarcity of human tissues.

Ovary

Women are born with two ovaries situated on either side of the uterus in the abdomen (Fig. 4.1a). The mature human ovary is approximately the shape and size of an almond and is the source of all eggs ovulated during a woman's reproductive life. A woman's ovary that is reproductively active is an extremely heterogeneous tissue containing follicles at all stages of development, corpora lutea (CL) and blood and lymph vessels (Fig. 4.1b). The follicle is considered to be the functional unit of the ovary, with each follicle containing a single oocyte (Fig. 4.1b).

The centre of the ovary contains numerous blood vessels that provide the ovary with all the nutrients and molecules required. These blood vessels are also responsible for the transport of gonadotrophins from the brain to the ovary and hormones from the ovary to the brain, essential for follicle development. These vessels are thus critical as a hormone transport mechanism for the hypothalamic-pituitary-ovarian axis (HPA) required for the regulation of follicle development and the menstrual cycle, discussed in Chapters 5–8. Blood vessels permeate the majority of the ovarian tissue with one significant exception; the basal lamina surrounding the granulosa cells. The outermost layer of each follicle, the theca cell layer comprising both the theca interna and externa, is vascular, whereas the inner granulosa cells, separated from the theca cells by a basal lamina, are avascular (Fig. 4.2a).

Unlike the majority of organs in the body, the ovary is extremely dynamic and under frequent

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

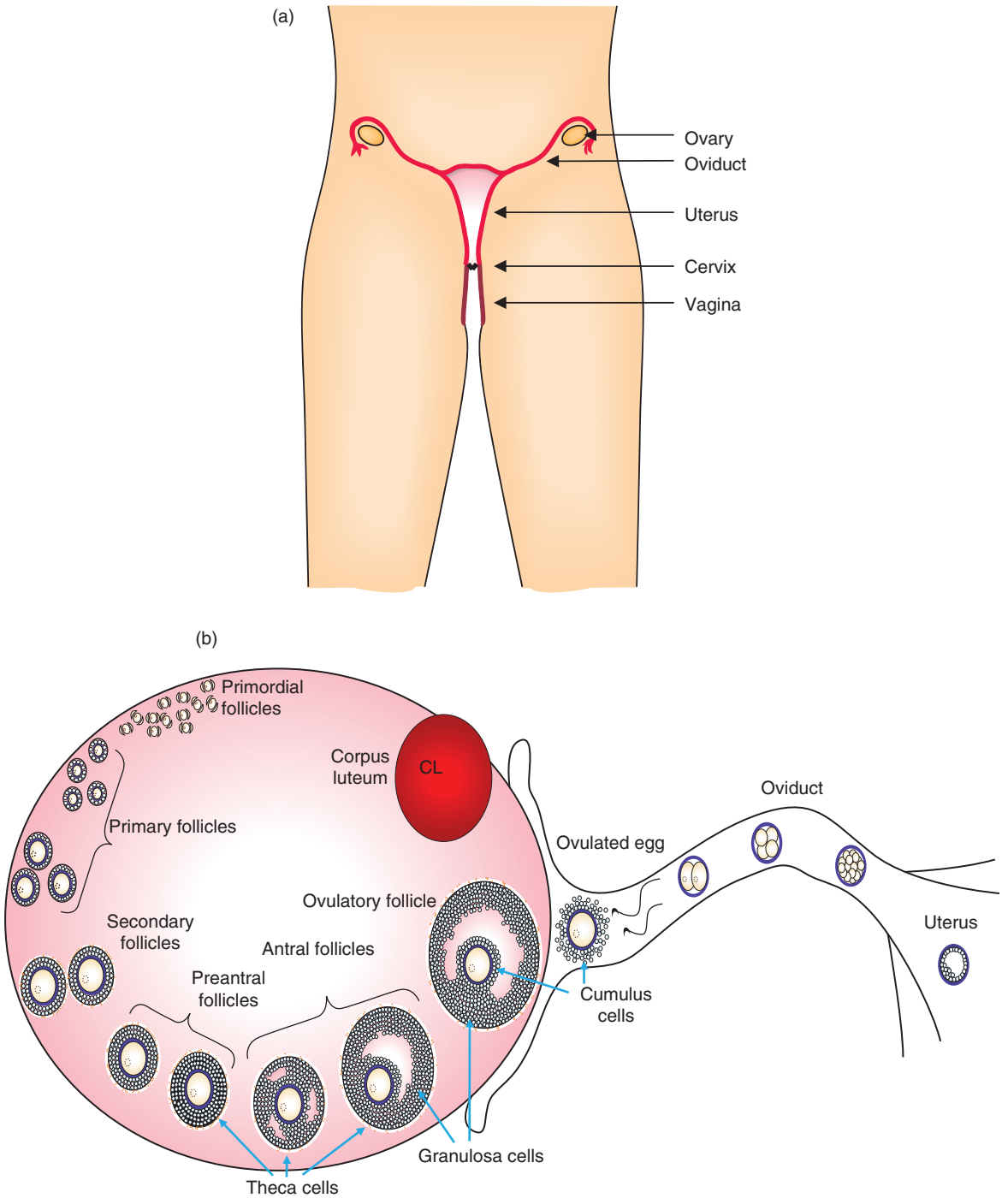

Figure 4.1 The female reproductive tract and organs. (a) The location of the female reproductive organs. (b) Ovary follicle development. Taken from Williams and Stanley (2011) [26].

reorganization. Follicles, which contain the all-important oocytes, develop from primordial follicles to primary follicles, then secondary follicles, preantral and antral follicles and eventually to large ovulatory antral follicles, otherwise known as Graafian follicles (Fig. 4.2a). In women, this takes approximately 6 months, in mice, approximately 3 weeks. Follicle and oocyte development will be discussed in greater depth in the second part of this chapter.

At the edge of the ovary are the meiotically quiescent primordial follicles and as they develop and increase in size they 'grow' into the centre of the ovary. There is some evidence that follicles migrate in humans and mice, although studies are inconclusive. In horses,

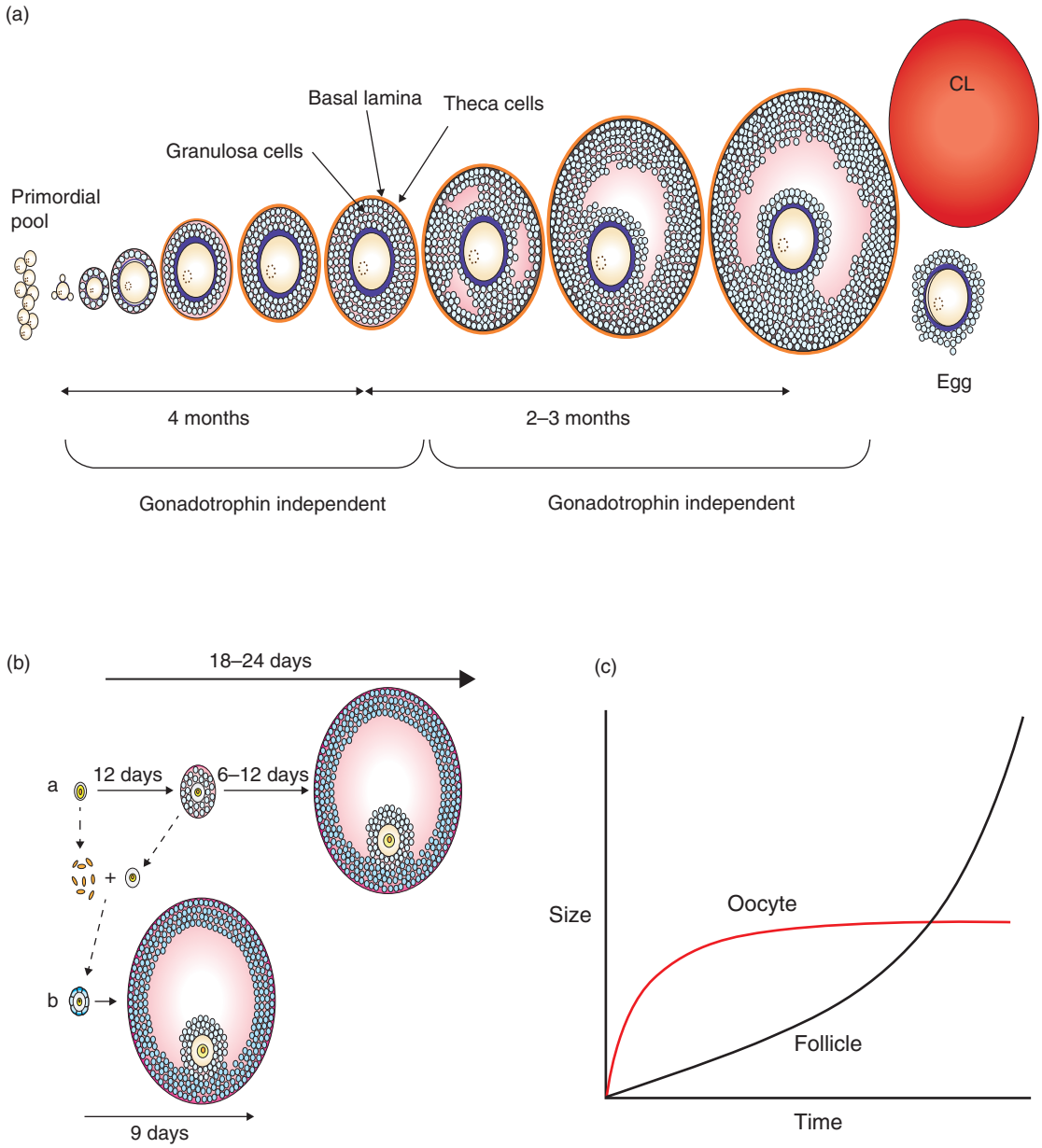

Figure 4.2 (a) Follicle development. (b) Oocyte orchestrates follicle development. (c) Relationship of follicle and oocyte growth.

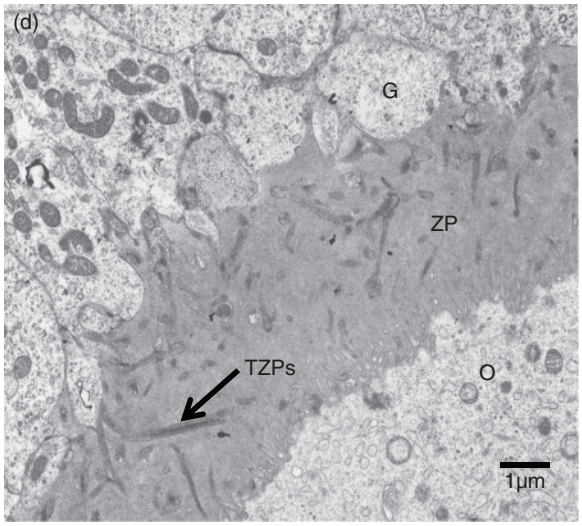

Figure 4.2 (d) Electron microscopy of oocyte-granulosa signalling via transzonal processes (TZPs) from the granulosa cells (G), through the zona pellucida (ZP) to connecting with the oocyte (O). Taken from Eppig, *et al*. (2002) and Williams and Stanley (2009) [4, 27].

follicles do indeed migrate since there is only one site of ovulation in these ovaries, and follicles need to position themselves at the appropriate location for ovulation.

In addition to follicles, the ovary also contains corpora lutea (CL). These structures develop via differentiation of the ovulatory follicle cells after ovulation (discussed in detail in Chapter 5). At the LH surge (Chapters 5 and 6), the theca and granulosa cells of the follicle differentiate and undergo luteinization. Thus, the entire follicle undergoes a functional change into a structure known as a corpora lutea (CL), which is the Latin term for yellow body. However, the term yellow body is misleading since the functional corpus luteum is a highly vascular organ and as such is a shade of red. The corpus luteum dominates the next phase of the cycle. The corpus luteum develops rapidly, growing as fast as the fastest growing tumour (personal communication, Dr Robert Robinson). When a functional corpus lutea is no longer required, it degenerates into a corpus albicans which is more yellow in colour. The molecular signals that regulate CL regression in women remain unclear.

The heterogeneity of the ovary, a small organ, provides a challenge to elucidating the function of many ovarian proteins. For example, to analyze the transition of follicles from the preantral stage involves collecting follicular cells for analysis. Collecting enough granulosa cells from a follicle at a specific stage of development for analysis is a challenge. For RNA analyses, this is not too difficult. However, the analysis of target proteins represents a significant challenge. Furthermore, in women we know that multiple follicles develop and yet only one will ovulate. To analyse the differences between the developing follicles requires comparative analysis of individual follicles. Furthermore, due to the small size of ovaries, analysis of protein or RNA from homogenized tissue does not take into account the day of cycle, stage and number of follicles present, the presence of CL or blood flow. Therefore, while representing an older laboratory technique, the use of histological sectioning and immunohistochemistry to analyse proteins is entirely appropriate since this allows analysis of different stages and tissues within follicles generated in vivo.

Oviduct

Once ovulated, the oocyte is referred to as an egg. It can also be referred to as a secondary oocyte owing to the fact that it completed second meiotic division at ovulation. The oocyte is ovulated into the abdominal cavity and is guided into the oviduct, otherwise known as the fallopian tube or uterine tubes. The oviduct consists of three sections: the infundibulum, the ampulla and the isthmus region. The infundibulum is the first section and receives the ovulated egg. The edges of the infundibulum are fringed with fimbria and these 'waft' the egg towards the opening of the fallopian tube. The egg enters the fallopian tube and begins its journey down the highly convoluted tube. The egg initially travels towards the isthmus – the site of fertilization. It is to here that the sperm migrate and where fertilization occurs (covered in detail in Chapter 11).

Since the egg has no means to propel itself, it is entirely dependent upon the surrounding environment for movement down the oviduct. Three mechanisms are involved in the unidirectional movement of the egg within the fallopian tube. The first is the secretion of oviductal fluid from the ciliated secretory epithelium that line the oviduct, resulting in the flow of fluid towards the uterus. These secretions also nourish the developing embryo. Second, the cells that line the oviduct have cilia which beat in a single direction ensuring the flow of fluid is unidirectional towards the uterus. Indeed, studies have shown that if a portion of the oviduct is removed and replaced in the opposite direction, the egg migrates in the opposite direction. Finally, the wall of the oviduct is muscular and contractions propel the egg onwards to the uterus.

While in the oviduct, the fertilized egg is supported by nutritional resouces provided by secretions from the secretory epithelium. Furthermore, recent studies have shown that the oviduct environment is critical to the development of the embryo and its future health as an adult due to epigenetic programming [1].

Uterus

After fertilization, the embryo progresses through clearly defined stages of development as it continues on its journey to the uterus. Here the embryo can implant and the uterus will nurture the developing fetus for the next 9 months.

The human uterus is the shape of a pear and about the size of a clenched fist, and is located low in the abdomen (Fig. 4.1a). The function of the uterus is to provide a supportive environment for development of the fetus. During fetal development, growth occurs within the uterus from the few cells of the hatched blastocyst to a 'full size' baby with an average weight of approximately 7 lb, demonstrating the uterus's incredible elastic properties to expand and stretch. In rare instances, the uterus can expand to extreme levels, for instance the largest singleton baby ever born weighed a staggering 23.12 lb. Moreover, the uterus can of course nurture multiple siblings. However, there is a limit to this capacity and multiple siblings usually result in preterm labour. Multiple siblings are also associated with many serious conditions and complications for adult health, as well as an increased risk of cerebral palsy and stillbirth. Consequently, singleton pregnancies are the best option for both mother and child.

The uterus contains multiple layers of tissue. The outermost layer of the uterus is a highly vascular layer known as the perimetrium. Inside this is the myometrium which is composed of smooth muscle. The muscle mass of the myometrium increases during pregnancy due to an increase in the length and number of muscle fibres. This is an important adaption in order to be able to produce the very strong contractions required for labour. Finally, the innermost layer of the uterus is known as the endometrium and generates the thick nourishing layer into which the embryo implants. The endometrium is composed of two sublayers: the *stratum functionalis* and the *stratum basalis*. The innermost layer, the *stratum functionalis*, proliferates during each cycle generating a highly vascular layer into which the embryo will implant. In the absence of implantation, the endometrium is shed at each menstrual cycle. The structure and function of the endometrium are dependent on the stage of the estrous cycle, with ovarian hormones regulating the lining of the uterus (discussed further in Chapters 5 and 6). The *stratum basalis* regenerates the *stratum functionalis* after each menstrual cycle.

Cervix

At the base of the uterus is the cervix which connects the uterus to the vagina (Fig. 4.1a). The cervix has numerous functions. The cervix secretes mucus, the constitution of which changes at different stages in the cycle as a result of hormonal regulation. During estrus, the mucus is thin and allows sperm to penetrate readily, whereas during other stages of the cycle, the mucus is thicker presenting a more hostile environment for sperm to pass, thus rendering the cervix almost impenetrable. The cervix also carries out an important function in the pregnant woman by helping to retain the fetus within the uterus. The cervix is also more mobile than might be imagined. Upon orgasm in the woman, the cervix dips onto the pool of semen within the vagina, promoting its passage within the reproductive tract to the site of fertilization.

Cervical cancer is common in women and results from infection by human papilloma virus (HPV). There are many different strains of HPV and these are common in the human population. Specific strains of HPV, namely HPV 16 and 18, carry particularly high risk for inducing cervical cancer. There are treatments available for this potentially deadly disease, initially in the early stages by removal of the cancerous tissue. Although tissue removal is required to remove the diseased tissue, repeated removal of cancerous tissue can result in the inability of a woman to carry a baby to term. One of the most important recent advances in the regulation of this disease is a vaccine against HPV. In the UK, there is a national programme to vaccinate all girls aged 12 to 13 against HPV which should significantly reduce the number of cases of cervical cancer in years to come.

Vagina

The vagina is a muscular canal lined with stratified squamous non-keratinizing epithelium. This canal connects the external reproductive organs with the internal organs. The penis ejaculates semen into the vagina during intercourse and it is the vagina through which a baby is born.

The vagina elongates during sexual arousal to accommodate the penis which subsequently deposits semen into the vagina at ejaculation. The vagina presents a relatively hostile environment to sperm for the majority of the estrous cycle. However, there is a more receptive phase at ovulation, when the mucus changes in response to hormonal stimuli. There is some evidence that the vaginal environment can also play a role in gender selection, with X or Y sperm more suitable to different pH. Interestingly, one risk factor for preeclampsia is the lack of acclimatization of the female to the partner's seminal fluid over several months [2]. This condition is highly prevalent in teenage mothers who tend to have shorter relationships with men prior to conceiving.

Oocyte development

The focus for the second part of this chapter is oocyte development. As stated earlier, the development of the oocyte and the follicle are inter-dependent. Until 1996, the oocyte was believed to be a passive cell at the whim of the follicle with oocyte division halted at meiotic prophase. However, in 1996, with the generation of mice lacking GDF-9, it was revealed that the oocyte plays a critical role in the development of the follicle [3]. Follicles in mice that were deficient in GDF-9, an oocyte-specific glycoprotein, developed only until the primary stage with follicles containing enlarged oocytes with single layer of granulosa cells. Elegant experiments in mice have revealed that the oocyte also has a role in the regulation of the duration of follicle development [4]. Mouse ovaries from newborn mice, containing only primordial follicles, and from mice age 12 days containing preantral follicles, were dissociated and oocytes and granulosa cells were collected separately. Follicles were re-aggregated using oocytes from the preantral follicles with the pregranulosa cells from the primordial follicles and the time of follicle development analysed. The duration for large antral follicles to be generated was determined by the developmental stage of the oocyte and not the granulosa cells (Fig. 4.2b). These experiments demonstrate that although the follicle supports and nurtures the development of the oocyte, this occurs in response to signals from the oocyte.

Oogenesis is the generation of the oogonia (described in Chapter 6) which exist as primordial germ cells in primordial follicles. These primordial follicles are finite in the newborn ovary and thus represent the total pool available to the woman for her entire life. Each primodial follicle takes approximately 6 months to develop into an ovulatory follicle, a Graffian follicle, which contain and ovulate a functional oocyte. However, since a woman is fertile for many years, there needs to be a constant supply of follicles and thus, throughout a woman's reproductive life, primordial follicles that have been resting in meiotic prophase begin to grow, resulting in a continuous supply of ovulatory follicles. The oocyte is unable to develop without the nurturing environment of the follicle, and vice versa. Consequently, the follicle is unable to develop without appropriate signals from the oocyte. Therefore, the following section will discuss both oocyte and follicle development.

Oocytes, known as primary oocytes prior to ovulation when the first meiotic division is completed, and ovulated eggs, are known as secondary oocytes until fertilization which initiates the completion of the second meiotic division, producing a zygote and polar body.

Follicle size

As follicles develop, their size increases. However, the increase in size is not continuous (Fig. 4.2c). Completion of the earliest stages of follicle development takes significantly more time than the latter phases of development. In addition, growth of the oocyte and the follicle are not equal or synchronous. The oocyte grows in size in a manner that is out of proportion to the growth of the follicle during the early stages of follicle development (Fig. 4.2c).

Primordial follicle growth resumption

Despite reference in the literature to the oocyte being 'frozen' at the primordial stage of development, the oocyte remains active during follicle development, and is also likely to be active in the resumption of development. Recent work investigating the regulation of primordial follicle growth resumption has indicated signalling pathways regulating follicle growth [5]. Such pathways appear to be initiated by growing follicles. However, primordial follicles clearly respond to these signals and are therefore most definitely not 'frozen'. The mechanisms that regulate the resumption of primordial follicle growth remain unclear. However, recent work has revealed a role for both Pten (phosphatase and tensin homologue deleted on chromosome 10) and Tsc1 (tumor suppressor tuberous sclerosis complex 1). Transgenic mice with targeted deletions of Pten, resulting in over-activation of

the phosphatidylinositol 3-kinase (PI3K) signalling pathway, or targeted deletion of Tsc1, which negatively regulates mammalian target of rapamycin complex 1 (mTORC1), caused all primordial follicles to resume growth, prematurely depleting the primordial follicle pool resulting in premature ovarian failure [6–9]. Therefore, although these two molecules have been identified as regulators of the primordial pool, working synergistically to maintain primordial follicles in their quiescent state, there still appear to be additional mechanisms in place that remain to be discovered. Furthermore, although this pathway may be involved in women who undergo premature growth of their primordial follicles, it is unlikely to be involved in women with POF in which ovaries were originally deficient in primordial follicle numbers.

Non-genetic approaches have also yielded insight. Some recent advances have indicated that the location of the follicles within the ovary may regulate the resumption of meiosis. In other words, the primordial follicles closest to the centre of the ovary tend to resume growth first, and therefore due to location, are exposed to different mechanical stimuli. Therefore, as those nearest the centre are removed from the primordial pool, those next to them will then be those nearest the centre and are thus next in line to resume growth. These theories arose from in vitro follicle studies where the density of the alginate the follicles were grown in affected follicle growth and viability [10]. This theory of mechanoreceptor regulation of follicle development is an interesting new approach and further work is needed to clarify these ideas.

Zona pellucida

At the primary stage of development, the oocyte begins to generate a zona pellucida, an extracellular matrix that is generated by the oocyte during follicle development. The proteins that comprise the zona are generated in humans, mice and rats exclusively by the oocyte. The mouse zona comprises three glycoproteins, whereas the human and rat ZP comprises four. This thick glycoprotein matrix surrounds the oocyte and is generated continually from the primary stage until ovulation, with additional zona glycoprotein secreted by the oocyte added to the ZP on the inside. This provides a challenge to the cellular connections that exist between the granulosa cells and the oocyte. These connections between the two cells, known as transzonal processes (TZPs), exist throughout follicle development, extending longer and longer as the zona gets thicker and thicker (Fig. 4.2d). Significant communication occurs between the oocyte and granulosa cells via these TZPs, including the prevention of meiotic resumption by the oocyte (discussed further in Chapter 5).

The zona pellucida has multiple functions. The most well-known functions are to protect the oocyte during and after ovulation, and to prevent multiple sperm fertilizing the egg. The zona appears to have additional functions *in situ* since mice generating oocytes lacking a zona due to the absence of a zona protein are infertile and fail to ovulate functional eggs [11]. However, mice generating a zona half as thick as a normal zona exhibit normal fertility [12]. Therefore we can infer that the structure of the zona has a role in the regulation of oocyte growth and development prior to ovulation.

Follicle development

Increase in follicle size is due initially to proliferation of the granulosa cells which occurs at all stages of follicle development. The granulosa cells synthesize and secrete estradiol, essential for follicle development. As the follicles develop to the secondary stage, surrounding stromal cells in the ovary align themselves around the edge of the follicle, which outlined by the follicle's basal lamina, differentiate into theca cells. The theca cells synthesize and secrete androgens; these androgens are the precursor for estrogens. The follicle continues to increase in size by proliferation of both the granulosa and theca cells. The next stage of development from a preantral follicle to an antral follicle requires the formation of an antrum. This antrum consists of follicular fluid and indicates the change in status of a follicle from a gonadotrophin-independent follicle to a gonadotrophin-dependent follicle. The composition of follicular fluid is carefully regulated and contains secretions from the granulosa cells, oocyte and also fluid that has entered the follicle by crossing the basal lamina.

The basal lamina is an extracellular matrix that exists between the granulosa cells and theca cells selectively restricting the molecules that enter the follicle based on size and charge. The basal lamina comprises different proteins, and the proportions of these vary with the stage of the cycle. Recent studies have demonstrated that alterations in the morphology of the follicle basal lamina are a good indicator of oocyte quality [13]. As the antrum increases in size, the cells surrounding the oocyte are induced to

differentiate from the mural granulosa cells into cumulus cells by secretions from the oocyte. The concentration of these factors decreases with distance from the oocyte and a diffusion gradient regulates the differentiation of these cells [14]. The final stage of oocyte development prior to ovulation is triggered by the LH surge. Binding of LH to receptors on the cumulus mass was previously thought to be the initiator of germinal vesicle breakdown. However, more recent studies have revealed that there is a specific mediator for this reaction: epidermal growth factors (EGFs). LH binds to the mural granulosa cells which stimulates the secretion of EGFs, which in turn bind to EGF receptors on the cumulus cells. This discovery reinforces how likely it is that future scientific endeavour will reveal significantly more insight into such processes.

Intrafollicular signalling

Signalling within the follicle between the oocyte, granulosa cells, theca cells and at certain stages of follicle development, cumulus cells, is exceptionally complex. This is due to the heterogenous nature of all of the tissues in the ovary, and the list of molecules involved grows longer by the year. This increase in understanding of the complexity of the interactions is emphasized by two reviews by Matzuk and Lamb [15, 16]. The number of molecules identified as having a role in follicle development and ovarian function increases dramatically, as can be seen in Figure 4.3 taken from two reviews by the same group published just 6 years apart. Furthermore, a comprehensive review of transgenic mouse models with altered follicle development further demonstrates the complexity of regulation [17]. Therefore it would clearly be impossible to discuss all of these factors here. Therefore, the intention here is to only select molecules required for specific functions which relate directly to the generation of a functional oocyte and direct the reader to scientific literature for further information.

Gap junctions

Connections between the granulosa cells and also between the oocyte and the granulosa cells are critical [18]. These communication channels use gap junctions which are established very early in follicle development. The oocyte and granulosa cells are linked by gap junctions assembled from connexin 37. These granulosa cells adjacent to the oocyte connect through the zona pellucida. It is these connections that maintain the oocyte in meiotic arrest as retraction of the TZPs induced by the LH surge results in meiotic resumption. The granulosa cells are connected by connexin 42. Gap junctions assembled from different connexins exhibit different permeabilities. Interestingly, elegant experiments have demonstrated that oocyte-specific connexin 37 is functionally replaceable by connexin 42 [19], thereby inciting the question why the two different proteins exist. Further studies will no doubt shed light on further refinements in the regulation of oocyte development.

Oocyte signalling

The oocyte, as previously demonstrated, has an active role in its own development and since this discovery, a variety of molecules generated and secreted by the oocyte have been the focus of intensive research. Growth differentiation factor 9 (GDF-9), bone morphogenetic protein-15 (BMP-15) and BMP-6 are such molecules. In addition to an individual function, GDF-9 and BMP-15 have also been demonstrated to function synergistically and different regions of each protein exhibit complex interactions. A further development intensifying the interest in these two oocyte glycoproteins was the discovery that heterogeneity of either GDF-9, BMP-15 or the BMP-15 receptor Alk6, was responsible for the long documented increase in ovulation rate in specific strains of sheep [21]. In a human study of twins, a loss of function mutation in GDF-9 was found in one case but also demonstrated clearly that alternate pathways are also involved in this phenotype [22]. Homozygous deletion of either oocyte glycoprotein results in sterility with ovarian hypoplasia [23–26] whereas in humans, defective BMP-15 and GDF-9 have been linked to premature ovarian failure. Therefore, since female fertility is restricted by the number of eggs ovulated, understanding the mechanisms that regulate fertility are clearly of great interest.

Oocyte health

The most important aspect of oocyte development is how it pertains to the generation of a healthy oocyte. The oocyte selected, ovulated and fertilized, is the cell that contributes all of the future embryo's organelles and cytoplasm while the sperm's contribution is purely genetic. Therefore, understanding how the ovulated egg is selected from all of the developing oocytes is of critical importance so that we can determine

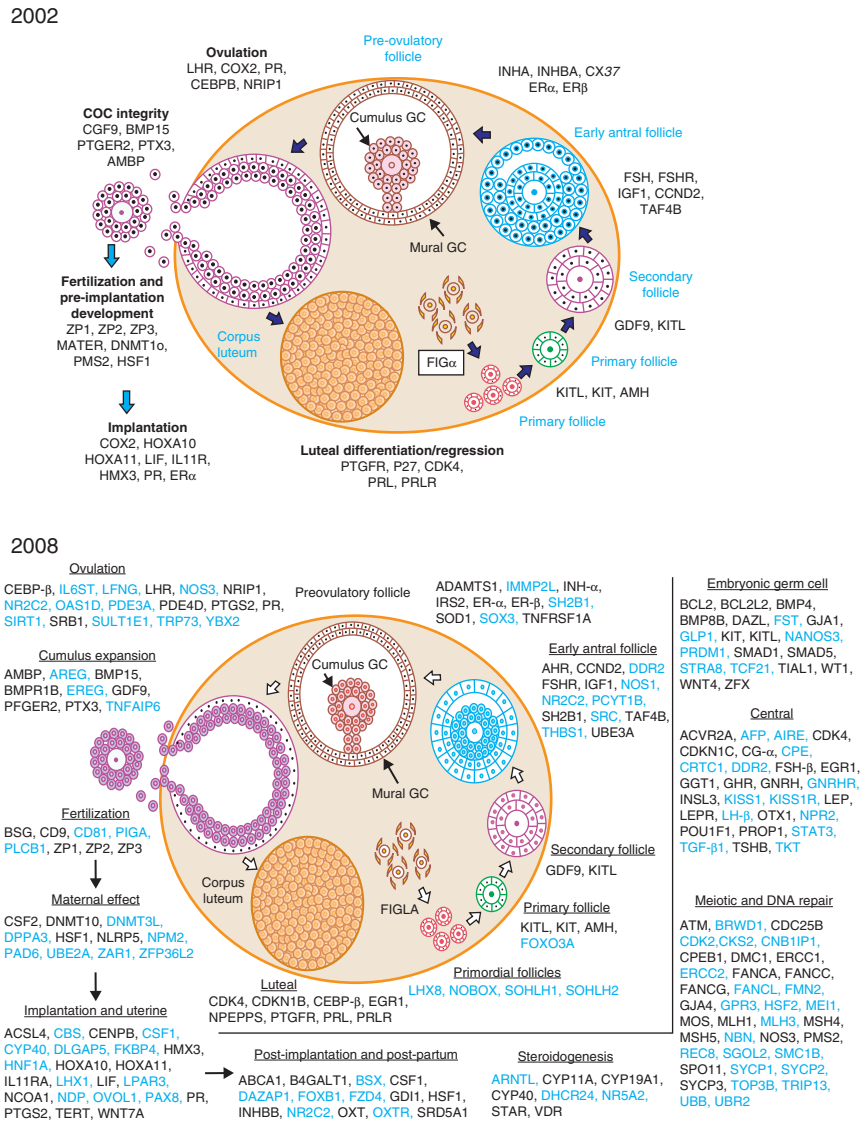

Figure 4.3 Illustration of the rapid discovery of molecules involved in the regulation of follicle development adapted from two reviews by the same authors six years apart. Taken from Matzuk and Lamb (2002, 2008) [15, 16].

which to select for assisted reproductive technologies (ART). In women undergoing natural cycles, just one oocyte is usually selected for ovulation, yet routine clinical techniques to support the development of multiple follicles using additional gonadotrophins result in numerous ovulations. Furthermore, most of these eggs are capable of fertilization and normal embryonic development – therefore, the burning question is to define the difference between the one oocyte that is naturally selected for ovulation and those that we can support to ovulation. Understanding what generates a healthy oocyte and being able to identify the 'healthy' from the 'not-so-healthy' is the holy grail of understanding oocyte development. This pinnacle is gradually being scaled with numerous advances in the assessment of oocyte quality. One example, discussed in detail in Chapter 28, is by analysis of the polar body, which reflects the genetic status of the oocyte. Using this new technique we can determine if an oocyte is aneuploid versus diploid, a cause of Down's syndrome. This pioneering technique enables defective oocytes to be screened out at an early stage, prior to fertilization, thereby ameliorating any ethical concerns. Further developments of techniques to assess oocyte quality,

such as analysis of the follicle basal lamina, will clearly assist in the future of ART.

Acknowledgements

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Chapter 5

Ovulation and regulation of the menstrual cycle

Farah El-Sadi, Anas Nader and Christian Becker

Introduction

The intricate mechanisms involved in the regulation of the menstrual cycle in humans are rather unique in the animal kingdom. While most viviparous animals undergo significant uterine changes solely in preparation for fertilization and implantation, the human endometrial lining is constantly in a state of flux. This dynamic state is borne out of the cyclical influences of a myriad of endocrine and paracrine events, which collectively produce the well-recognized stages of the menstrual cycle. In the absence of implantation, these changes culminate in the shedding of a significant portion of the endometrial lining. This heralds menstrual bleeding, and with it, the beginning of a new cycle.

Until recently in human history, the female menstrual cycle was still shrouded in a mystery that generated ample superstitious beliefs and practices. Although we have witnessed a leap in our understanding of the menstrual cycle at the molecular level, there is much research to be done to fine-tune this understanding. In this chapter, we will briefly examine what is currently known about the phases of the menstrual cycle, what governs them and how the various key players in this process interact to produce the seamless transition from one cycle to the next.

Conventionally, the commencement of the menstrual cycle is designated as the first day of menstruation (Fig. 5.1), a period during which the endometrium is shed in response to hormonal withdrawal. In a slight departure from this convention, our discussion of menstrual cycle regulation will begin with exploring the process of follicular recruitment and maturation in response to the hypothalamic stimulation of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release. At mid-cycle, the surge in LH induces ovulation. During the ensuing latter part of the cycle, the levels of progesterone peak, thereby inducing what is known as 'secretory changes' in the endometrium, consisting of glandular enlargement, stromal cell swelling and an increase in blood supply. In the absence of pregnancy, estrogen and progesterone withdrawal causes endometrial breakdown resulting in menstruation.

Unlike the stereotypical unfolding of events during the menstrual cycle, the duration of each cycle is highly variable. In women who experience regular menstrual cycles, the average length of cycles is largely dependent on age. For women ages 15–19 years, the range of cycle length (from 5th to 95th centile) is 23–35 days. This range was noted to decrease to 23–30 days in women between the ages of 40 and 44 years [1]. Given that the vast majority of the normal population cycle length falls within 22 and 35 days, a regular cycle length of 21 days or less is defined as polymenorrhea, while a regular cycle length of 36 days or more is defined as oligomenorrhea. If no menstrual bleeding occurs within at least six months it is termed amenorrhea.

Hypothalamic and pituitary contribution

Ovarian hormonal secretion is under tight regulation from the hypothalamic-pituitary-ovarian axis (Fig. 5.2). Menarche, or the onset of menstruation, is marked by an increase in the amplitude and frequency of the gonadotrophin-releasing hormone (GnRH) pulses (see Chapter 7). This stimulates the release of LH and FSH from the anterior pituitary, thereby inducing the release of estrogen from the ovaries.

An intricate mechanism of 'negative feedback' and 'positive feedback' operate at the level of the

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

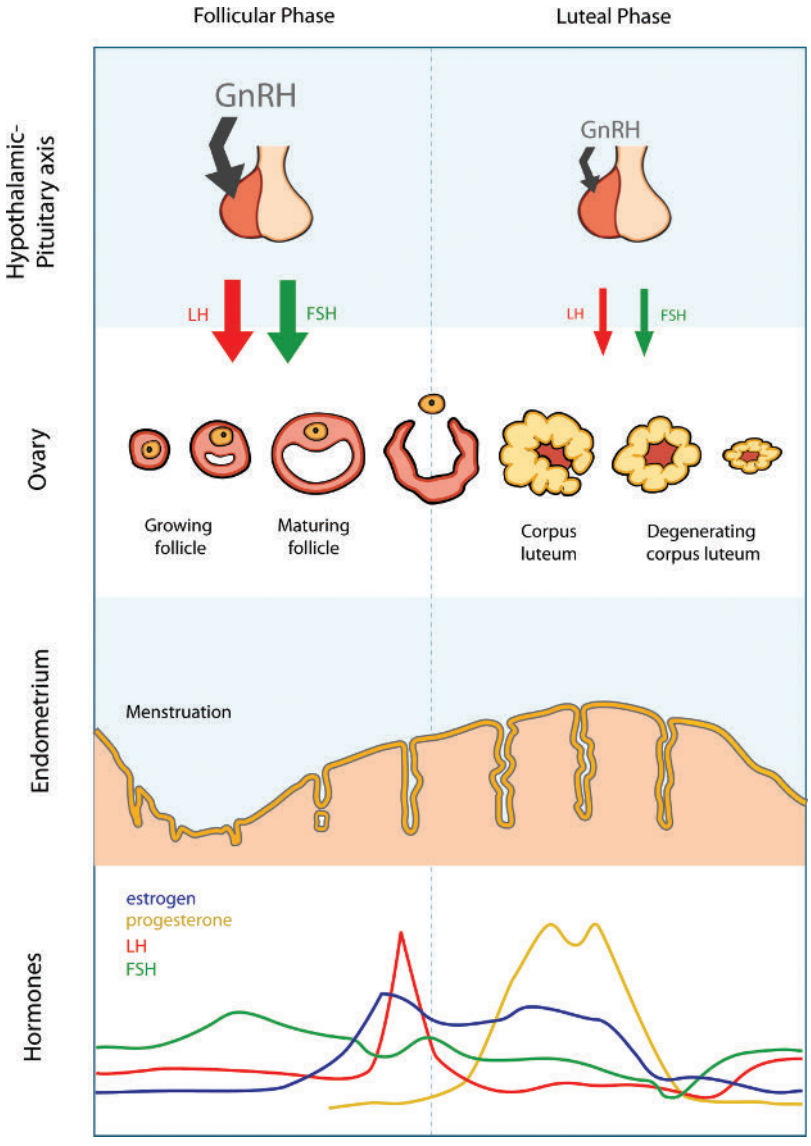

Figure 5.1 LH/ FSH and estradiol/ progesterone surges with corresponding structure of the endometrium.

hypothalamus and the pituitary to regulate the release of GnRH, LH and FSH respectively. The functional interactions between the different components of the follicular apparatus result in steroid hormone production, which in turn feeds back to the hypothalamus and the pituitary [2]. More specifically, ovarian estradiol production initially suppresses FSH release through a negative feedback effect on the pituitary. This results in the maturation of a single follicle. As the mature follicle continues to release estradiol, a positive feedback effect is generated causing a surge in the level of LH, thus heralding the beginning of the ovulatory phase of the cycle.

A brief overview of folliculogenesis

Unlike the testicles, the ovaries exercise their exocrine and endocrine function cyclically over a confined period of time during the life span; namely between puberty and the menopause. Ovarian function is served by their layered structure consisting of an outer cortex lined by a germinal epithelium, and an inner medulla comprising connective tissue and blood vessels (Fig. 5.3). The basic functional unit of the ovary is represented by an ovarian follicle, composed of an oocyte surrounded by granulosa and theca cells. The primordial germ cells migrate at week 5–6 of

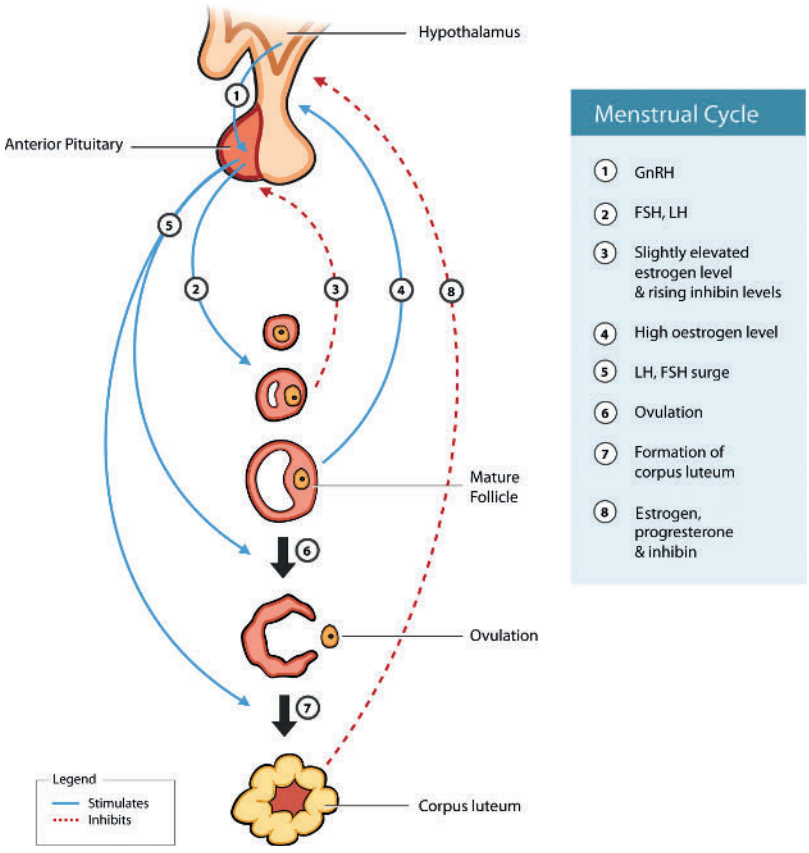

Figure 5.2 LH/ FSH feedback loops from estradiol, along the hypothalamic-pituitary-ovarian axis.

gestation to the genital ridge. Substantial mitotic activity occurs within these cells until 20 weeks of gestation. This results in the development of 6–7 million germ cells in both ovaries. The germ cells within these follicles remain arrested at prophase I of meiosis until ovulation. More than 99% of these 'primordial follicles' undergo atresia (apoptosis) until menopause, and only about 400–500 follicles will ovulate during a woman's reproductive life [3]. Atresia can affect follicles at all stages of development. The fate of each follicle, be it atresia or further growth, is determined by a poorly understood interaction between the circulating levels of steroids and gonadotrophins, the stage of the cycle itself, and various intra-ovarian factors [3].

The process of maturation of such primordial follicles into preovulatory follicles is known as folliculogenesis. This process is continuously occurring within the ovaries from puberty to menopause such that there are follicles at different stages of development within the ovaries at any point in time. Although the process of maturation from the primordial to the preovulatory phase has been well described, the mechanism responsible for the initiation of follicular growth and the mechanism allowing variable timings to the primordial follicular activation remain illusive.

Folliculogenesis begins with the formation of primordial follicles around the seventh month of embryonic development. The oocyte within the follicle is surrounded by a layer of flattened follicular (squamous) cells. The transformation of the flattened squamous cells into cuboidal cells characterizes the transition from a primordial follicle to a primary follicle. A basal membrane known as the membrane of Slajvanski covers the cuboidal cells [4].

The proliferation of the cuboidal follicular cells to form a second layer around the oocyte marks the transition from a primary to a secondary or pre-antral follicle. At this stage, differentiated epithelioid cells from the stromal fibroblasts are recruited via oocyte-secreted signals. These cells form the internal and external thecal layers (theca interna and externa). This cohort of pre-antral follicles takes

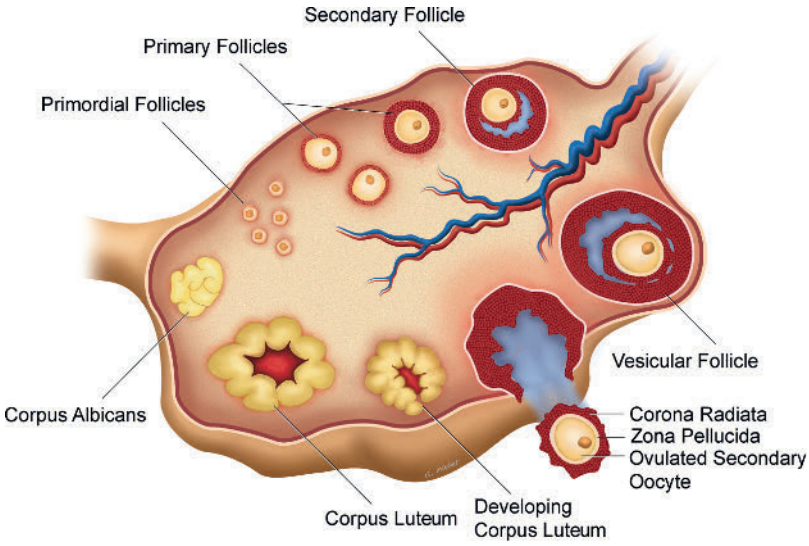

Figure 5.3 Ovary with follicles at different stages of maturation.

approximately 85 days to reach the preovulatory size [3]. It is also of note that follicles which enter the pre-antral stage at mid-cycle coincide with the preovulatory release of FSH and LH. Theca cells produce androgens when stimulated by the release of LH from the pituitary. Since entry into the pre-antral stage can occur at any point during the menstrual cycle, follicles whose theca interna maturation does not coincide with the preovulatory period will become atretic [4]. From the primordial stage to the pre-antral stage, growth and maturation occur independently of gonadotrophin release. However, once the theca interna commences its epithelioid differentiation, the follicle is able to respond to gonadotrophin stimulation due to the presence of LH and FSH receptors in the theca interna and the granulosa respectively [3].

The tertiary follicular formation is dependent on the development of an antrum in the granulosa cell layer; a fluid-filled cavity adjacent to the oocyte containing nutrients and steroid hormones (Fig. 5.3). Antral follicles with the highest proliferation rate contain the highest estrogen/androgen ratio. Furthermore, the cells forming the granulosa cell layer differentiate into distinguishable subtypes, the most notable being the cumulus oophorus, which project into the follicular cavity around the oocyte. If selected as a result of the rise in FSH, this 'awakened' oocyte would be recruited into the follicular phase of the next menstrual cycle [3].

Stages of the menstrual cycle

The follicular phase

Selection of the dominant follicle

The process of selecting a dominant follicle begins with recruitment of a maximum of five follicles per ovary at the beginning of the follicular phase [4]. The recruitment of follicles coincides with a rise in FSH plasma levels, which effectively rescues them temporarily from atresia. This is in part due to recruited follicles being larger in diameter (2–5 mm), making them more sensitive to FSH as evidenced by the increased aromatase activity [5]. More FSH receptors are produced as a result, thus further sensitizing granulosa cells to the effects of FSH, leading to further estrogen production from androgens. In addition, this stimulates the expression of LH receptors on the granulosa cells. However, at mid-follicular phase, the FSH levels begin to decrease, and a single 'dominant' follicle is selected.

The suppression of FSH levels is due to increased central inhibition by rising estrogen levels originating from the recruited follicles. Reduced FSH levels lead to a reduction in aromatization of androgens into estrogens within the follicles and an increased production of endocrine and paracrine factors such as inhibin-B tumor necrosis factor (TNF) and anti-Müllerian hormone (AMH). Only the dominant follicle is able to adapt to this hostile environment due to higher FSH receptor levels and its increased vascularity in the

theca cell layer, allowing the preferential delivery of the gonadotropin to the follicle. Hypertrophy of the theca interna and its increased vascularity continues until the LH surge occurs [6].

The structure of the preovulatory follicle is crucial in understanding the process of steroidogenesis preceding the LH surge. The full structure is depicted in Figure 5.3. The wall of the preovulatory follicle consists of (starting from the outside) an outer ovarian surface epithelium (OSE), tunica albuginea, theca externa, theca interna, a basal lamina and a granulosa cell layer containing a germ cell and its surrounding cumulus cells [7]. Anatomically, the theca interna is the most vascularized layer of the ovarian follicle [8]. It is a source of progesterone and androgen precursors necessary for estrogen synthesis. The granulosa cell layer, normally avascular until follicular rupture, is where follicular aromatase activity (stimulated by FSH) results in the synthesis of estrogen during follicular development [9]. The fluid-filled follicular antrum contains a melange of follicular secretory products such as estrogen, progesterone and inhibin, in addition to serum transudate [7]. It is the hormonal secretory products of these cell layers that induce the LH surge, thereby initiating the process of ovulation.

The LH surge

The onset of ovulation is remarkably variable both between women, and for the same woman from one cycle to another. However, within a cycle, the timing of ovulation can be predicted with reasonable accuracy on the basis of the LH surge. This surge precedes ovulation by approximately 34–36 hours [10], as it is a crucial step in the cascade of events leading to follicular rupture. Since low levels of estrogen have an inhibitory effect on LH secretion, the LH surge is thought to be initiated by the mid-follicular rise in peripheral estradiol concentrations [11]. As the preovulatory follicle matures, increasing amounts of estrogen are produced. A sustained exposure to and the attainment of a threshold level of at least 200 pg/ml of estrogen lead to the induction of the characteristic LH surge. This effect is crucially facilitated by the simultaneous central effect of a preovulatory rise in progesterone [12]. The combined effect of elevated estrogen and progesterone levels leads to an increased frequency in central GnRH pulsatility, which favours LH secretion from the pituitary. The preovulatory progesterone plays another important role in the sequence of events leading up to a successful ovulation. Progesterone has been found to affect a significant mid-cycle FSH peak (Fig. 5.1), which is currently thought to be a necessary step in ensuring an adequate granulosa-cell response in the form of producing sufficient LH receptors [10].

The pulsatile GnRH release brought on by the increasing quantities of estrogen, and by progesterone to a lesser extent, results in the down-regulation of GnRH receptors [7]. Moreover, the rising concentration of progesterone and the diminishing estrogen signal further contribute to weakening the effect of the GnRH signal, eventually suppressing gonadotropin release.

Ovulation

The onset of ovulation is heralded by the LH surge and the smaller but equally important mid-cycle FSH surge. Both events initiate a cascade of follicular responses, which eventually culminate in finalizing the oocyte maturation process, thus freeing the oocyte from its follicular attachments, thereby resulting in follicular rupture and ovulation.

The LH surge induces the progression of meiosis from its arrested state in the premature oocyte and the luteinization of the granulosa cells, ultimately leading to the physical release of the oocyte and its surrounding cumulus mass [10]. These events are normally prohibited in the presence of local factors within the oocyte such as oocyte maturation inhibitor (OMI) and luteinization inhibitor (LI). Yet, in the presence of LH, cyclic AMP activity is induced, and these local inhibitors are overcome in favour of promoting oocyte maturation and luteinization [13].

Hormonally, the process of luteinization causes a decrease in the aromatization of androgens into estrogen within the granulosa cells, which can only occur in the presence of LH receptors on the granulosa cells. The decreased estrogen production is coupled with an elevation in the levels of progesterone, resulting from increased cholesterol side chain cleavage to produce pregnenolone, a precursor of progesterone. In addition, there is an increased production of 3β-hydroxysteroid dehydrogenase [14], an essential enzyme in the catalysis of progesterone from its precursor.

The preovulatory LH surge induces a transformation of some granulosa cells into luteal cells. The resulting progressive rise in progesterone levels around the time of ovulation has a dual role: on one hand, the rising progesterone has a central effect on the pituitary as it exerts a negative feedback to cease

the LH surge. On the other hand, progesterone plays a vital role in follicular rupture by stimulating a range of proteolytic enzymes, which act on collagen particularly within the thecal layer, thus disrupting its structural integrity and allowing its disintegration [10]. One of the most significant proteolytic enzymes present within the preovulatory follicle is plasminogen activator (PA). Two forms of PA exist within the follicle prior to ovulation; tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) [7]. These factors convert plasminogen in the follicular fluid to its active form plasmin. Plasmin can either act directly on the follicular wall to weaken it, or it can lead to the activation of several matrix metalloproteinases (MMPs) such as the conversion procollagenase into collagenase, which in turn digests the follicular wall at its apex resulting in ovulation. Progesterone is only one of many factors identified as activators of proteolytic enzymes such as PA. The LH surge also contributes significantly to the production of plasminogen activators (tPA and uPA) from granulosa and theca cells [15]. Furthermore, tumour necrosis factor (TNF) produced in apical endothelial cells within the ovulatory follicle has also been shown to stimulate ovarian PA [16].

The destabilization of the follicle is also promoted by the small peri-ovulatory surge in FSH brought on by the rising levels of progesterone. This process is achieved through the FSH-induced stimulation of hyaluronic acid production, a ubiquitous carbohydrate polymer that promotes cellular movement and proliferation. As a result of such stimulation, the cumulus cells surrounding the oocyte disperse and expand in order to create an oocyte-cumulus unit that can float within the antral fluid prior to rupture.

Finally, the gonadotropin surge preceding ovulation has also been shown to stimulate the production of cyclo-oxygenase in the granulosa and theca cells [17] leading to a significant increase in prostaglandin synthesis. Prostaglandins were found to play a central role in follicular rupture. This is supported by the finding that inhibition of prostaglandin synthesis with the administration of nonsteroidal anti-inflammatory drugs can prevent ovulation [18]. The contribution of prostaglandins to follicular rupture is multifactorial. They have been shown to bolster the effect of proteolytic enzymes on the digestion of the follicular wall by freeing them from other components of the wall [19]. Moreover, they have been implicated in ovarian smooth muscle contraction, thereby facilitating the expulsion of the oocyte-cumulus mass from the follicle [10].

The luteal phase

The word 'luteal' stems from 'lutein', which is a xanthophyl carotinoid pigment. Derived from the word *luteus* (Latin for yellow), this pigment normally accumulates in granulosa cells prior to follicular rupture. Chronologically, the luteal phase begins with the formation of the corpus luteum from an ovarian follicle and ends with either luteolysis or pregnancy if fertilization and implantation are achieved. This phase is characterized by a marked surge in progesterone levels unparalleled by any other stage of the menstrual cycle. Other hormones such as estradiol and inhibin-A are also produced, albeit to a lesser extent, by the corpus luteum. The collective actions of progesterone, estradiol and inhibin-A on the pituitary finally culminate in the suppression of further gonadotropin release, thereby allowing a new cycle to begin and new follicular growth to occur.

Central to the events occurring during the luteal phase is the transformation of the ovarian follicle into a corpus luteum, a term which translates literally to the 'yellow body'. The corpus luteum develops from granulosa and theca cells after ovulation. Therefore, at least two distinct cell types can be seen to make up the mature corpus luteum. The smaller luteal cells respond to LH by increasing their secretion of progesterone. This is likely due to their origin as thecal cells. Conversely, large luteal cells contain receptors for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), thereby facilitating the luteolytic action [20]. The process of luteinization is now identified as being an outcome of the ovulatory LH surge.

Following the ovulatory LH surge, luteinized granulosa cells produce vascular endothelial growth factor (VEGF) and angiopoietins, thereby inducing angiogenesis in the granulosa layer [21]. VEGF expression results in the proliferation of endothelial cells, which facilitates the neovascularization of the corpus luteum. On the other hand, angiopoietins play a role in determining the stability of the formed vascular walls. Vascular stabilization is achieved by the binding of angiopoietin-1 to its endothelial Tie-2 receptor [22]. By the mid-luteal phase, maximum vascularization levels are achieved. The functional significance of vascularization during the luteal phase becomes apparent when one considers the role of the corpus luteum in

steroidogenesis. Progesterone production by luteal cells is dependent on the availability of its substrate, namely LDL-cholesterol [20]. Vascularization of the granulosa cell layer allows the efficient delivery of LDL to the luteal cells, which possess LDL receptors that facilitate the binding and internalization of this substrate [23]. Besides vascularization, the increased delivery of cholesterol is also dependent on the activation of protein kinase A (PKA), which is induced by luteotropic hormones such as LH. Once PKA is activated, it is thought to stimulate progesterone production through increasing the transport of cholesterol to P-450 side chain cleavage enzyme complex [24]. Consequently, the peak of progesterone production is reached 8 days after the onset of ovulation. In addition, the extensive capillary network formed during luteal development ensures that the high metabolic demands of the corpus luteum are met, especially as it can consume two to six times more oxygen per unit weight compared to the heart, kidney or liver [25].

The exact mechanisms responsible for the involution and degeneration of the corpus luteum (also known as luteolysis) 9–11 days after ovulation are still unclear. While this process is the default outcome in primates in the absence of pregnancy (therefore the corpus luteum demise has to be rescued by pregnancy hormones if fertilization occurs), non-primate mammalian animals need to actively induce the degeneration of the corpus luteum if fertilization does not occur. The processes in primates leading to the default outcome are multifaceted and, as mentioned earlier, remain largely illusive. Nonetheless, two events have now been established to occur during luteolysis. Progesterone synthesis and secretion ceases, and the cells that make up the corpus luteum are lost [20].

There is ample experimental evidence implicating local estrogen in the induction of luteolysis [26]. Estrogen is thought to trigger the release of oxytocin from the pituitary [27], which in turn induces the release of $PGF_{2\alpha}$ from the uterus [28]. In some species, $PGF_{2\alpha}$ mediates luteolysis partly through stimulating the production of endothelin-1, a potent vasoconstrictor [29]. Endothelin-1 also induces apoptosis via the release of tumour necrosis factor-α (TNF-α) [30]. The resulting apoptosis of endothelial cells reduces the density of the capillary network within the corpus luteum. Consequently, blood flow is markedly reduced, thereby depriving luteal cells of the necessary nutrients and substrates to resume steroidogenesis [31]. The reduction in the density of steroidogenic cells, which normally produce tissue inhibitors of metalloproteinases (TIMPs), leads to the production of proteolytic enzymes such as MMPs [32]. MMPs induce tissue remodelling within the corpus luteum by acting on and degrading the extracellular matrix [33]. Luteolysis also triggers significant vascular breakdown through the action of angiopoietin-2 on its endothelial receptor Tie-2 [10].

In the event of a successful fertilization of the ovum, the corpus luteum structure is actively 'rescued' from the default demise. Human chorionic gonadotrophin (hCG), which is structurally very similar to LH, is produced by the newly formed embryo and acts to maintain steroidogenesis and the vascular system within the corpus luteum, thereby preventing its degeneration. The rescued luteum continues to produce progesterone until placental steroidogenesis is established by the tenth week of gestation [34].

Towards the end of the luteal phase, the demise of the corpus luteum triggers a marked decline in the production of progesterone, estrogen and inhibin-A. This in turn lifts the negative feedback inhibition on the pituitary's release of gonadotropins. The increased release of LH and that of FSH are not equal in magnitude. The FSH increase is greater than that of LH during the luteal-follicular transition. Inhibin-A has long been noted to suppress pituitary FSH release [35], therefore the reduced inhibin-A production brought on by luteolysis causes a marked and selective increase in FSH [36]. The predominant increase in FSH is bolstered by the change in the frequency of the pulsatile secretion of GnRH following the reduction in steroidogenesis. These effects, along with the general reduction in steroidogenesis, result in the increased production of FSH from the anterior pituitary, and the subsequent selection and development of a preovulatory follicle for the next menstrual cycle.

Structural changes within the endometrium during menstruation and subsequent phases of the cycle

The structure and dynamic function of the human endometrium present a well-coordinated system. It is intriguing that a monthly lesion and the resulting wound healing do not cause scarring with subsequent infertility. The structure of the endometrium is dynamically responsive to the changes in the female hormonal milieu. The stages of the menstrual cycle, which are

marked by varying levels of endocrine and paracrine signals, can be seen mirrored in the changing structure of the endometrium throughout the cycle (Fig. 5.1). Simply put, the endometrium proliferates and thickens under the influence of estrogen, and differentiates under the influence of progesterone, while the withdrawal of progesterone results in endometrial shedding and menstruation (Fig. 5.1). These structural changes are facilitated through the bi-layered composition of the endometrium, both of which form target tissues for steroid hormones. The shedding of the top functional layer during menstruation is followed by regeneration from the underlying basal layer.

The hormonal influence on the structure of the endometrium is transmitted through changes in receptor expression. The pattern of steroid receptor gene expression varies throughout the menstrual cycle [37]. This variability in the expression of sex steroid receptors, namely progesterone receptor (PR), estrogen receptors (ERα and β) and androgen receptor (AR), is also under hormonal influence. For example, the expression of ERα and PR is up-regulated under the influence of estradiol during the follicular phase and down-regulated under the influence of progesterone during the luteal phase [38].

The structural changes of the endometrium are not exclusive to its bi-layer, but extend to the structure of the endometrial vessels as well. Endometrial blood vessels form spiral arterioles in the upper two-thirds of the functional layer [39]. The composition of the vessel walls is also dynamic, comprising endothelial cells, basement membrane and surrounding perivascular cells expressing smooth muscle actin. Menstrual bleeding is induced by destruction of the spiral vessel walls under the influence of progesterone withdrawal.

The perivascular stromal cells, under the influence of progesterone, synthesize the matrix supporting the basement membrane. The mechanism of this effect has been elucidated through in vivo observations of progesterone-mediated suppression of proteolytic enzymes such as collagenases and MMPs [40]. This effect is further enhanced in the presence of TIMPs, whose synthesis is stimulated by progesterone [41].

Menstruation begins on the first day of the follicular phase; the ensuing vaginal bleeding is of variable duration and can typically last between 4–5 days. The progesterone and estrogen withdrawal initiated at the end of the luteal phase causes a cascade of intracellular events leading to the destabilization of the basement membrane with subsequent shedding of the functional tissue layer. Increasing vasoconstriction of spiral arterioles leads to hypoxia resulting in vascular hyperpermeability and apoptosis. This mechanism appears to be caused by the expression of stromal prostaglandins. Leucocytes and red blood cells extravasate into the perivascular space and stroma. Progesterone withdrawal appears to induce expression of enzymes such as matrix metalloproteinases (MMPs) and suppress their natural inhibitors Tissue Inhibitor of Matrix Metalloproteinases (TIMPs) resulting in digestion of the endometrial tissue and extracellular matrix. Although the source of MMP production is currently unknown, it is thought that the invasion of the endometrium by leucocytes, especially neutrophils and monocytes, provides a reservoir of such lytic enzymes [42]. The ultimate result is menstrual bleeding during which the tissue and blood of the functional layer are lost while in the basal layer under the influence of rising estrogen levels, regeneration begins for the new menstrual cycle [43].

Summary

The menstrual cycle is a complex process, which is tightly regulated by changing levels of estradiol and progesterone. The largely estradiol-dominated follicular phase marks a significant proliferation of stromal and glandular epithelial cells in the functional layer of the endometrium just after menstruation. This is followed by a surge in LH secretion inducing ovulation. After the ejection of the oocyte, the corpus luteum forms out of the theca and granulosa cells of the follicle, giving rise to high progesterone levels. During the luteal phase high progesterone levels drive the secretory transformation of the endometrium necessary for the implantation of a blastocyst approximately 6 days post ovulation. If pregnancy occurs, human chorionic gonadotropin (hCG) secreted by the trophoblasts will support the corpus luteum, ensuring sufficient progesterone levels until 8–9 weeks of gestation when the placenta becomes the main source of progesterone. If implantation fails the corpus luteum will disintegrate into the corpus albicans. The sharp drop in both progesterone and estrogen levels results in contraction of endometrial blood vessels leading to local hypoxia and enzymatic digestion of the functional layer of the endometrium and ultimately menstruation.

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Chapter 6

Key events in early oogenesis affecting oocyte competence in women

Geraldine Hartshorne

Introduction

This chapter aims to outline key topics from early oogenesis that may directly affect oocyte competence and hence eventual fertility. Following a brief overview of the topic, more detailed descriptions of the timeline of oogenesis, female meiosis and the origins of maternally inherited genetic abnormalities, and oocyte growth will be presented.

The human oocyte is an extraordinary and unique cell type, originating during fetal life. Before birth several key events take place in the nascent oocytes that have the potential to affect not only the fertility of the baby girl as she grows to adulthood, but also the quality and genetic content of the oocytes that will become her children.

During the mid trimester of pregnancy, at an early stage in their development, oocytes in the fetal ovary become arrested in meiosis. In this state, they cannot replicate and so individual oocytes survive as distinct cells for an exceptionally long period of time. To illustrate this point, the oocyte that formed me was already present in an immature form when my maternal grandmother was carrying my mother, in the 1920s. My own daughter in utero carries oocytes that may one day become my grandchildren. This longevity is one of a number of interesting properties of oocytes.

Prolonged survival of immature prenatal oocytes is dependent upon their becoming surrounded by specialized somatic cells, pregranulosa cells, that support the oocyte's metabolism. Together, the oocyte surrounded by a single layer of pregranulosa cells forms a discrete and stable unit known as a primordial follicle. These primordial follicles comprise the so-called resting state of follicle development. Primordial follicles are the most numerous follicles in the ovary at all stages from birth until the menopause and are normally located within the cortex of the ovary, i.e. the outer 1–2 mm of the surface. However, only about a third of the nascent fetal oocytes survive beyond birth and thereby contribute to the oocyte pool from which mature oocytes and the next generation may eventually derive.

The arrested oocyte in its primordial follicle is highly resilient to the changing environment of the female body and retains its unique properties, despite the female's progression from baby to girl to adult, hormonal changes and menstrual cycles, illness and injury, size and weight changes, glut and famine, pregnancy, childbirth and breastfeeding. A model calculating the rate of utilization of primordial follicles based upon histological specimens shows that follicle utilization is relatively stable among women aged less than 38 years, with acceleration at older ages. Under normal circumstances, an age-related proportion of primordial follicles remains until the menopause [1]. Although total follicle numbers cannot be measured directly in live women, most women have clinically measurable parameters of fertility that fall within a predictable age-related range, such as age at menopause, and responsiveness to gonadotrophins. Only radiation, gonadotoxic drugs (possibly including cigarette smoking), surgery affecting the ovaries, or genetic abnormality (e.g. Turner's syndrome XO) are known to profoundly deplete primordial follicle numbers in women [2–4]. In experimental animals, genetic modification of apoptotic cascade genes (e.g. bax and bcl-2) and factors controlling initiation of follicle growth (e.g. Tsc2, PTEN) are now shedding light on the mechanisms of early follicle growth control [5].

An individual primordial follicle persists, sometimes for decades, until the day when its growth

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begins, known as 'activation'. Early follicle growth proceeds slowly and the starting point is not easily identified. Once the follicle is activated, it becomes more vulnerable to the effects of paracrine and endocrine factors and increasingly stringent competition from other follicles that have also begun to grow. The initial growth of the follicle is locally controlled by factors within the oocyte, in concert with the granulosa cells. These stimulate and control the replication of the granulosa cells to form a solid ball comprising multiple layers, surrounded by a basement membrane. In closely coordinated fashion, there is an increase in size of the oocyte and production of the zona pellucida, a proteinaceous layer that remains in place until after fertilization and early embryo development. Although many follicles begin to grow around the same time, a progressively smaller number can be supported at any given stage beyond activation. Follicles are selected for continuing development or atresia by a process of competition. Follicles that cease growing and undergo atresia usually exhibit a form of granulosa-cell failure, often characterized by increasing numbers of apoptotic granulosa cells and eventual loss of oocyte viability [6]. This is in contrast to prenatal folliculogenesis, where the oocytes are usually the leading cell type to undergo apoptosis, possibly due to the number of somatic granulosa precursor cells being limiting.

As the follicles grow larger, paracrine feedback among follicles may affect their growth, for example, Anti-Mullerian Hormone (AMH) is produced by growing follicles and exerts inhibition upon the growth of other small follicles. This interfollicular communication has been demonstrated in vitro by experiments where follicles that are initially similar are placed in direct contact with each other. Such paired follicles always grow at different rates. Systemic factors, such as circulating hormones, also start to influence the follicle, once its cells have developed the appropriate receptors and post-receptor response mechanisms. For the selected population of follicles that survive and grow to a diameter of around 500μm, clinical interventions to promote or inhibit follicle growth may start to become effective. By this time the follicle is no longer a solid ball of cells, but contains a fluid-filled intercellular space called an antrum and a surrounding layer of thecal cells recruited from the ovarian stroma, outside the basal membrane (Fig. 6.1). From about 2 mm diameter, the small antral follicle becomes visible by transvaginal ultrasound scanning. The numbers of small antral follicles in the early follicular phase of a menstrual cycle are sometimes used as an indicator of potential ovarian responsiveness to gonadotrophin stimulation in this cycle (e.g. for IVF treatment), or as an indirect assessment of the ovarian reserve [7]. These small antral follicles are sensitive to follicle stimulating hormone, having developed functional FSH receptors during their growth, so clinical administration of FSH can promote their further growth and development. In contrast, at the time of writing, there are no clinical interventions that target the primordial follicle pool directly, even though many crucial aspects of

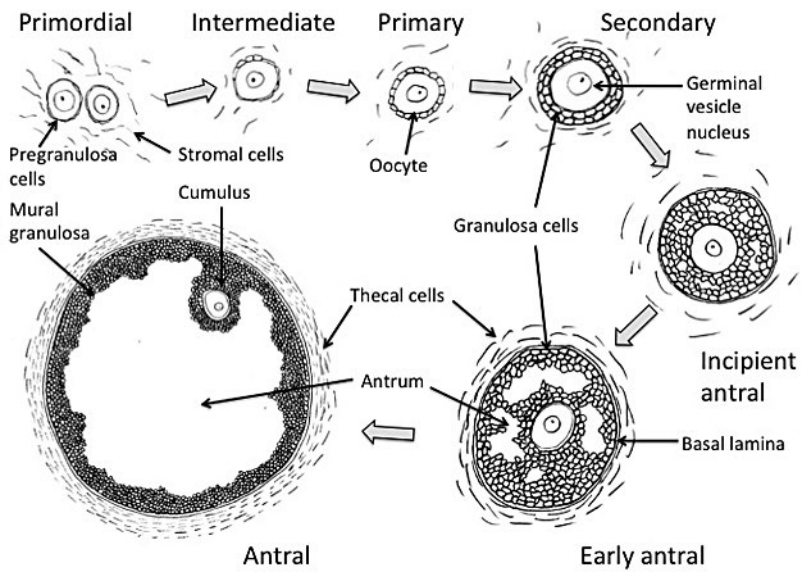

Figure 6.1 Diagram of stages of follicle growth.

fertility and successful reproduction depend upon early events in oogenesis. However, methods to produce clinically useful oocytes from follicle growth in vitro or in an animal host are now in prospect [8, 9].

Timeline of oogenesis

Many events underpinning female gamete competence occur in the prenatal ovary, many years before the onset of fertility (Fig. 6.2). Figure 6.2 provides a summary diagram, highlighting the timescale and interrelationships of key milestones in prenatal oogenesis, including meiosis, folliculogenesis and apoptosis.

The germline precursor cells that eventually result in gametes are called 'primordial germ cells'. These diploid cells are evident in the yolk sac, i.e. outside the main body of the embryo, at around 24 days post coitum (5–6 weeks of gestation). In mice, it is known that the primordial germ cells are descendents of cells found at a particular location during gastrulation in mice. Similar data are not available in humans, but it is likely that the germline is kept separate from the somatic components of the developing embryonic disc at an early stage. The reasons for this are not known, but might include, for example, facilitating differential imprinting of the germline. Primordial germ cells then move from the yolk sac to the developing gonads by a process of migration. During the migration process, many cells are lost from the population, and reasons for their loss may include misdirection, apoptosis or too slow progress. However, other cells divide during the journey and the net effect is for a large increase in population size during this time. Once they arrive at the ovary, at 6–7 weeks of gestation, the primordial germ cells lose their migratory properties but continue to divide by mitosis. They are known at this stage as oogonia. The term oocyte is not used until the diploid cells have entered meiosis; then they are correctly called oocytes.

Oocytes have first been identified from around 10 weeks of gestation in humans, when some of the oogonia initiate meiosis (Fig. 6.2). Those nearest the

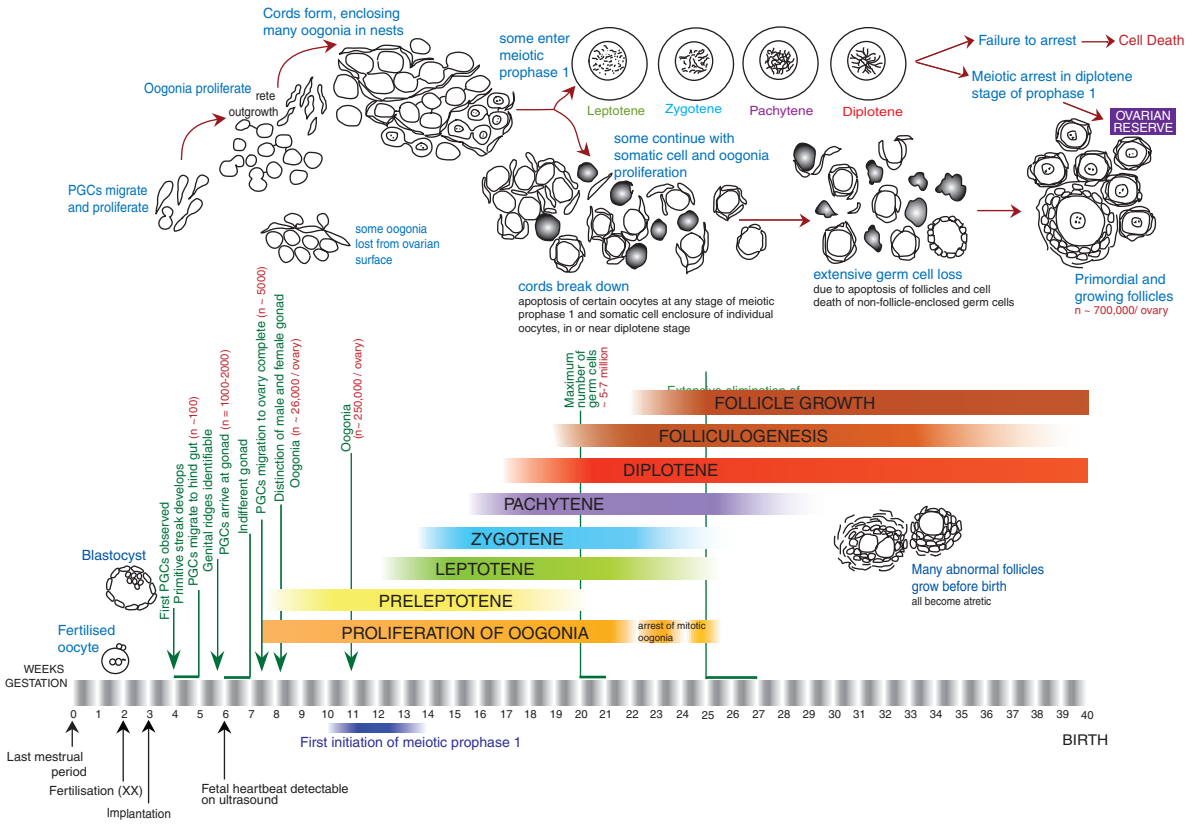

Figure 6.2 Timeline for human prenatal oogenesis. Figure reprinted from Hartshorne, Lyrakou, Hamoda, Oloto and Ghafari. Oogenesis and cell death in human prenatal ovaries: what are the criteria for oocyte selection? *Mol Hum Reprod* **15** (2009): 805–19 by permission of Oxford University Press, Human Reproduction and the European Society of Human Reproduction and Embryology.

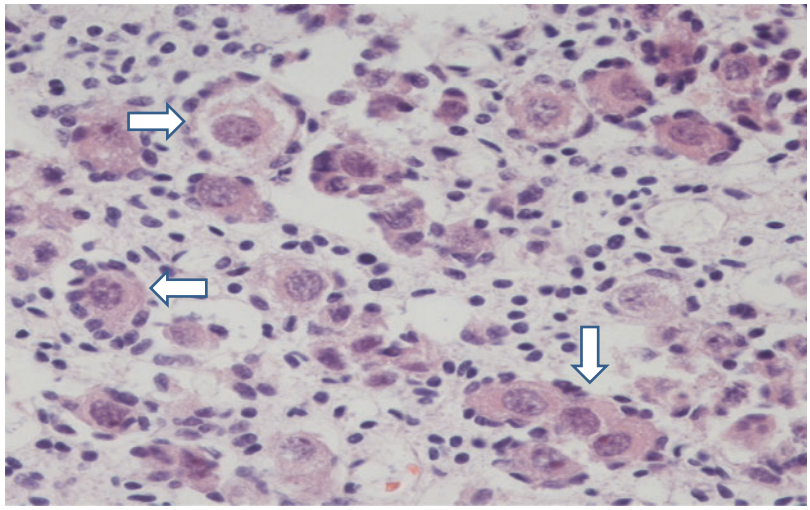

Figure 6.3 Follicle development in a human fetal ovary aged 23 weeks gestation. Vertical arrow shows three, possibly four, oocytes in a 'nest' with somatic cells bordering the periphery. Left arrow shows a primordial follicle surrounded by somatic pregranulosa cells. Right arrow shows an enlarged oocyte in a follicle that has started to grow. Histological wax section stained with haematoxylin and eosin. Figure kindly provided by Sabreen Mahmoud Ahmed, Warwick Medical School.

ovarian medulla enter meiosis earliest and continue to lead the pool during later developmental stages too. Oogonia enter meiosis over a period of several weeks, until approximately 24 weeks of gestation. Individual oocytes differ greatly in the rate at which they proceed through meiosis, and some oogonia, perhaps a quarter, fail to enter meiosis at all. These are believed to undergo mitotic arrest and are removed from the ovary eventually by apoptotic mechanisms; however, debate remains as to whether some may persist into adult life with capacity to initiate meiosis and generate oocytes by the process of 'neo-oogenesis' [10]. This is a highly controversial claim which has undergone intense challenge in the literature, and to date has not been proven [11].

Meiosis is the specialized cell division that eventually results in gametes that have a haploid chromosome constitution, discussed in more detail later. However, prenatal oocytes do not achieve full gamete formation at this prenatal stage. Instead, they become arrested in an immature state early in meiosis, in first meiotic prophase. Such oocytes are incompetent to undergo maturation or fertilization. At approximately 30μm diameter, they are small in comparison with a mature oocyte of ~ 115μm diameter. Prenatal oocytes are initially associated with other oocytes developing at a similar rate, in clusters known as 'nests', believed to be derived from the same oogonium. The nests break down as many of these small oocytes become surrounded by somatic, pregranulosa cells to form primordial follicles, identifiable in some fetal ovaries from 14 weeks' gestation at the earliest, but becoming more common from around 18 weeks of gestation (Fig. 6.3). Oocytes that do not arrest, or do not associate with somatic cells, are unable to sustain further development and are mostly eliminated before birth. From the middle of gestation (week 20) extensive apoptosis takes place among the oocytes, resulting in the loss of more than half of them. As apoptosis continues, most primordial follicles arrest and become what is known as the 'follicle pool' or 'ovarian reserve'. These primordial follicles remain in this 'quiescent' state, located in the cortex of the ovary, for an indefinite period during which the oocyte remains arrested, and the follicle retains a single layer of flattened pregranulosa cells. In contrast, some of the nascent primordial follicles in the fetal ovary do not arrest but grow further, resulting in primary, secondary and antral follicles before birth. Many abnormal follicles are also formed and these often grow before birth or during childhood [12], thus still being eliminated from the selected population that will contribute to the adult's eventual fertility.

Once the intense folliculogenesis and apoptosis of gestation have resolved, the resulting ovary contains a large population of primordial follicles, together with smaller numbers of follicles at stages up to the antral stage. From birth to the menopause, a small proportion of primordial follicles will become activated each day and begin to grow. However, follicles that grow before puberty always become atretic. Only after puberty, when the hypothalamo-pituitary-gonadal axis has matured, providing an appropriate endocrine environment to support full follicular development, can any follicles progress to Graafian stages and ovulate their oocyte. These ovulatory follicles, at most one

or occasionally two per month of adult life, represent the tiny minority, selected by a process of intense competition. The fate of the vast majority of follicles is atresia. Inevitably, therefore, the oocytes that give rise to mature gametes and contribute to subsequent generations are those which arrested successfully during prenatal development and formed a competent follicle that remained arrested for many years, initiated growth at an opportune moment and successfully outcompeted other follicles developing at the same time. The ultimate purpose of this complex process of follicle growth is to deliver a competent female gamete for fertilization. However, the process of oogenesis is also complex and there is no guarantee that the resulting gamete will be competent, even when follicle growth has proceeded optimally.

Female meiosis

The process of meiosis involves first duplication of the DNA in the 23 pairs of chromosomes, and then two halvings to result in a haploid chromosome complement. A diagram of the meiotic process is presented in Figure 6.4. It is important to understand the manner in which meiosis takes place, as this underpins many of the fundamental errors in embryo development occurring in human preimplantation embryos and in common abnormalities arising in human pregnancies, such as trisomies (for example, Trisomy 21 Down's syndrome, the commonest chromosomal disorder in humans). Errors during female meiosis are the greatest source of such anomalies [13]. A sound knowledge of meiosis also provides the basis for safe and accurate application and interpretation of preimplantation genetic diagnosis (PGD) and screening (PGS) techniques in human clinical embryology, for example, polar body biopsy and aneuploidy detection.

In the prenatal period, oocytes complete only the first part of meiosis, namely meiotic prophase I. The normally developing cohort of oocytes becomes arrested in the 'diplotene' stage towards the end of meiotic prophase I. At this stage the oocyte nucleus is

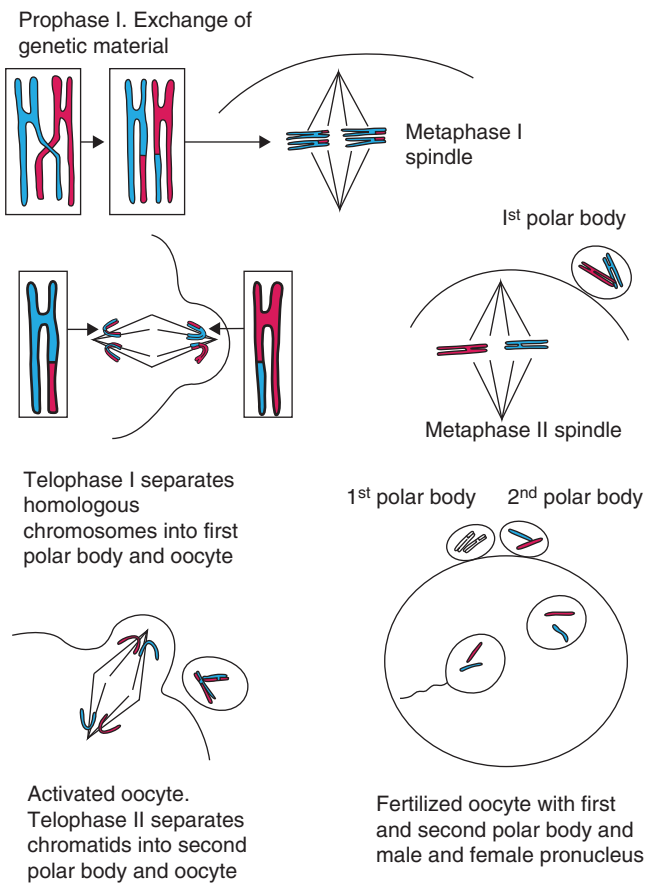

Figure 6.4 Diagram of chromosome movements during female meiosis. Recombination of maternal and paternal DNA takes place during meiotic prophase I, and oocytes subsequently undergo meiotic arrest at the diplotene stage, towards the end of meiotic prophase I (germinal vesicle stage). Shortly before ovulation, when the oocyte resumes meiosis, pairs of homologous chromosomes align on the spindle at metaphase I. Chromosomes are separated at telophase I, resulting in the first polar body and an oocyte that progresses rapidly to the metaphase II stage, at which point the second meiotic arrest takes place. If the oocyte is activated, for example, by a fertilizing sperm, the metaphase II spindle progresses to telophase II, resulting in the ejection of the second polar body. Note the different chromosomal contents of the first and second polar bodies of the fertilized oocyte.

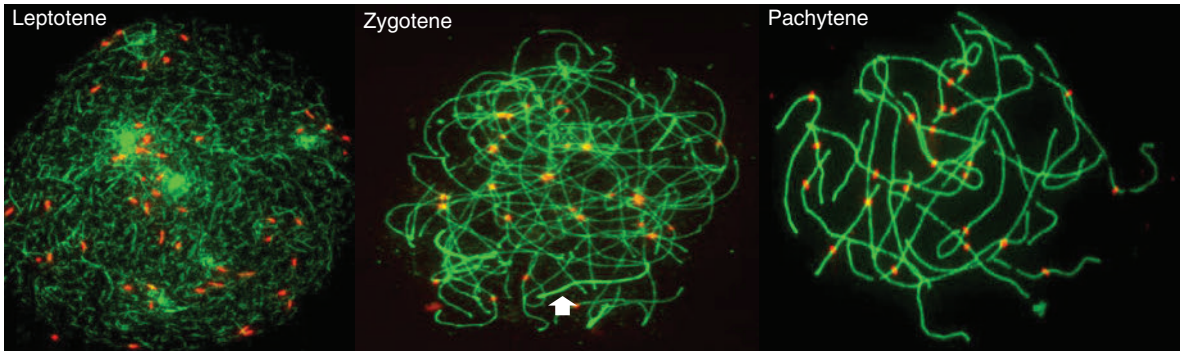

Figure 6.5 Stages of meiotic prophase I in human fetal oocytes. Axial elements in human oocytes from the second trimester of pregnancy were highlighted using a fluorescently labelled antibody. Axial elements are part of the synaptonemal complex, the protein backbone that holds chromosomes together during meiotic prophase I. At the leptotene stage, fragments of stained axial elements are evident. At the zygotene stage, longer elements are present and some of them have begun to synapse shown by thicker areas of synaptonemal complex (arrowed). At the pachytene stage, all homologous chromosomes have synapsed completely along their lengths. The synaptonemal complexes break down during the diplotene stage, so this stage is not shown here.

visible and characteristic, usually containing a single prominent nucleolus. The nucleus is known as a 'germinal vesicle' (GV), so this stage is commonly known to clinical embryologists as the germinal vesicle or GV stage. This arrested chromosomal configuration persists for all the time that the oocyte is in a primordial and non-atretic growing follicle. The surrounding granulosa cells are instrumental in maintaining the oocyte's arrest in this stage of meiosis over the duration of follicle growth, in addition to supplying nutrition and paracrine signalling molecules. Eventually, oocyte maturation from meiotic prophase I arrest (GV) to a second arrest in meiotic metaphase II (metaphase II, MII) takes place in response to the mid-cycle surge of luteinizing hormone, originating from the pituitary gland some 24–36 hours prior to ovulation. The mature oocyte is arrested in metaphase II and is normally ovulated in this stage of meiosis. However, the stage of meiosis of ovulated oocytes may vary for different species.

Meiotic prophase I comprises four stages: leptotene, zygotene, pachytene and diplotene. These are clearly distinguishable when the synaptonemal complexes, the protein backbone that holds the chromosomes in their meiotic prophase I configurations, are highlighted with specific marker antibodies (Fig. 6.5). However, much of the earlier literature in this field uses histological preparations, which are more challenging to interpret, resulting in wide discrepancies among data sets. Differences have also resulted from considerable variations in the fetal materials available for study.

In the leptotene stage, the chromosomes start to condense and homologous chromosomes move together. A specific conformation known as a 'bouquet' brings the termini of chromosomes into proximity at one side of the nucleus. How this is controlled is uncertain. The homologous chromosomes begin to synapse (adhering together progressively along their length), which marks the entry to the zygotene stage. As zygotene continues, chromosomes synapse for increasing lengths, identifiable as thicker regions of synaptonemal complex markers. During zygotene, the chromosomes must initially be prone to tangling as synapsis proceeds; however, when resolution is achieved, the result is an oocyte in which each of the homologous pairs is fully synapsed along the whole of its length. This is the pachytene stage, which is the most readily identifiable stage of meiotic prophase I.

Mainly at the pachytene stage, recombination between adjacent chromatids of different homologues takes place. This is the point at which the genes resident on the homologous chromosomes originally received from the male and female parents of the individual reciprocally exchange or 'cross over'. This results in genetic recombination and reshuffling of genes for the next generation of gametes, a particularly important stage and prone to errors. Each tetrad must have at least one chiasma, or crossing over point, in order to ensure that later separation of the homologous chromosomes takes place correctly. There is also evidence that the crossing over points are non-randomly distributed, and that the chances of

incorrect separation of chromosomes increase as chiasma location moves proximally or distally [13]. The number of chiasmata varies, but there is evidence that oocytes developing normally contain more chiasmata than those with abnormalities in synapsis of homologous chromosomes [14].

When the pachytene stage is complete, the oocytes progress to diplotene. At the diplotene stage, the synaptonemal complexes that have held the oocyte chromosomes in formation break down, and the chromosomes remain attached by their chiasmata only. The oocytes assume a stable conformation of a membrane-bound large nucleus, containing usually a single or sometimes two nucleoli, which is the germinal vesicle (GV) nucleus mentioned above. The DNA is dispersed, and transcription can take place. Although recombination takes place at this early stage in meiosis, the recombined products do not separate from each other until the ejection of the polar bodies during oocyte maturation and fertilization, several years later.

Abnormalities arising during meiosis

The relatively high prevalence of numerical chromosomal abnormalities in humans has been considered a major contributor to our species' relatively low fertility, and the possible mechanisms of origin have been the subject of extensive research. As a result, it is widely believed that most numerical chromosomal disorders in humans arise from the process of female meiosis. In particular, problems with abnormal separation of chromosomes as the first polar body is emitted have long been considered the primary mechanism, while errors at other stages also occur, including chromatid separation and second polar body formation, male meiosis, and mitosis of embryonic blastomeres. The foundations for the abnormalities originating in the oocyte are laid during the female prenatal stages, but come to fruition only during oocyte maturation and fertilization.

Many prenatal oocytes never complete the pachytene stage because of errors in synapsis, or perhaps an abnormal initial chromosome complement. Severe abnormalities are lethal, resulting in cells that are incapable of further development and sooner or later are eliminated by apoptosis. Different points in meiotic prophase I have been proposed as target stages for oocyte elimination; however, it seems that apoptosis may take place at any stage in meiotic prophase I.

For oocytes in which recombination abnormalities arise during the pachytene stage, it seems likely that the oocyte could continue to survive depending upon a certain level of cellular functions and the support of the granulosa cells. This is because problems with crossing over and chiasmata numbers laid down before birth may not become critical until many years later when oocytes mature from the diplotene stage of meiotic prophase I, to the metaphase stage of meiotic prophase II, just a few hours prior to ovulation and fertilization.

At the telophase stage of meiotic prophase I, the chromosomes are separated from their homologues along the meiotic spindle, becoming allocated to the gamete or the first polar body. Spindle competence is also crucial for normal segregation. The spindle is a highly energy-dependent structure, abnormalities of which can be induced by toxins, ageing and oxidative stress. If separation of a pair of chromosomes fails to occur, known as non-disjunction, then both the oocyte and the polar body will contain an incorrect number of chromosomes, resulting in an abnormal gamete. Other related telophase mechanisms causing abnormal numbers of chromosomes have also been described, such as premature chromosome separation and anaphase lag [13]. Similar segregation errors may also arise at telophase II, which occurs after sperm penetration of the oocyte and during fertilization.

The chances of numerical chromosome abnormalities in oocytes increase with female ageing, with important impacts upon society. An age-related decline in oocyte quality, including an increase in genetic abnormalities and cytoplasmic deterioration, accounts for the poorer reproductive outcomes that are prevalent in women towards the end of their reproductive years, when compared with a younger population. The poorer outcomes include lower chances of pregnancy, an increased chance of miscarriage and an increase in the occurrence of chromosomally abnormal offspring. Various interesting ideas have been proposed to account for this, including spindle abnormalities, oxidative stress, reduced mitochondrial performance, checkpoint leakiness, production line theories and selective delay of abnormal forms. Unfortunately, these topics are too broad to cover here [15].

The true incidence of genetically abnormal mature oocytes in humans is unclear because so few of them are available for study, and most of these originate from infertile patients undergoing clinically stimulated cycles with abnormally high levels of gonadotrophins,

resulting in the collection of oocytes that would not normally have reached ovulation. However, current estimates are around 20% [13]. This appears to be a high level of inefficiency at the end of a highly stringent oocyte and follicle selection procedure. Perhaps some underlying evolutionary benefit underlies the high proportion of abnormal oocytes formed, but to date it has not been identified.

Oocyte growth

The oocyte and the pregranulosa cells are a metabolically linked unit, with multiple cellular projections from the granulosa cells ending in gap junctions at the oocyte surface, mediating transmission of a range of signalling molecules and nutrients. This close association is essential for optimal growth control; however, the growth of the oocyte can be dissociated from the expansion of granulosa cell numbers, or even the close cytoplasmic linkage with granulosa cells, suggesting that early oocyte growth may be initiated from the oocyte itself. As mentioned above, for an immature oocyte in a primordial follicle to become a mature female gamete, it must increase its diameter from 30μm to approximately 115μm. This is equivalent to a 450-fold increase in volume. Early oocyte growth occurs while the oocyte remains meiotically arrested. An early growth spurt takes place in close coordination with the earliest stages of follicle growth. As the follicle grows to a diameter of ~124μm, a very tight relationship exists between the oocyte nuclear size, the oocyte size and the follicle size, with nuclear-cytoplasmic ratios maintained, suggesting a closely controlled process [16]. The oocytes in this histological study were considered fully grown at a diameter of 80μm, but even accounting for shrinkage during fixation, it seems likely that they were smaller than expected. The research material in this study was infant ovaries, which may have affected the response, since smaller oocytes have been noted in prepubertal ovaries of various species [17]. The later stages of oocyte growth have been little studied but interestingly, enlargement of oocytes in vitro during culture and maturation has also been reported [18].

Also during very early stages of growth, the zona pellucida starts to be formed; however, its location, between the granulosa and oocyte cell surface, does not appear to impede the expansion of size of the oocyte or the continuity of granulosa cell/oocyte communication via cellular projections. This suggests that its molecular structure differs at this stage, being more flexible and extensible than the multiply crosslinked structure evident in mature oocytes. The zona pellucida proteins are also closely involved with the earliest stages of follicle growth, for example, ZpA contributing to oocyte expansion in mice [19].

In humans, oocyte diameters after ovarian stimulation may vary, but in our hands, mature oocytes collected for ICSI treatment ranged between 112 and 119μm diameter [18]. It has been shown in many species that the functionality of the oocyte accrues stepwise with increasing size, such that thresholds for particular functions may be ascribed. For example, mouse oocytes with a diameter of < 60μm are incompetent to undergo GV breakdown (the first visible step of oocyte maturation), but larger oocytes normally have this competence. However, competence to mature to metaphase II and undergo subsequent development occurs progressively over the range 65–75μm oocyte diameter in mice. Similar stepwise acquisition of competence is reported in human oocytes, but with some disagreement over the precise threshold values.

Growth of the oocyte is accompanied by a range of cytoplasmic changes [20]. For example, the number of mitochondria increases from around 10 in primordial germ cells to around 100,000 in mature oocytes. The mitochondria are an important source of energy for maturation and fertilization, and have a range of other signalling roles. The growing oocyte also acquires reserves of mRNA in a closely controlled programme. Selected mRNAs are stored by masking them with a polyA tail that prevents their translation until the tail is removed at a key point in the developmental schedule. Some mRNAs are maintained until after fertilization during the period leading up to global embryonic genome expression during cleavage divisions. In contrast to the small, stable primordial oocyte in the GV stage, the oocyte approaching maturity is large, complex and unstable. Its functionality depends critically upon a balance of metabolism that is energy intensive and cannot be sustained for a long period. Mature human oocytes have a functional life of at most one day, after which deterioration is inevitable.

Conclusions

Oocyte quality critically underpins the likelihood of conception and reproductive outcome. Poor oocyte

quality due to advancing maternal age is one of the most intractable problems in assisted conception treatment. Many of the fundamental properties of the oocyte originate from prenatal events and are therefore not amenable to modification via interventions in fertility clinics. The best that can be achieved is optimized nurture of oocytes to maintain their developmental potential, where it exists, and accurate selection of the most competent from among those available.

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Chapter 7

Regulation of gonadal function

Nicolas Vulliemoz and Christian Becker

Introduction

Reproduction is a fundamental component of human physiology. Its regulation necessitates precise interplay between the hypothalamus, the pituitary gland and the gonads. In the female, gonadotropin-releasing hormone (GnRH) stimulates release of the two gonadotropins, follicular stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary gland. These pituitary hormones are secreted into the systemic circulation and reach the ovaries where they not only stimulate folliculogenesis but also stimulate secretion of ovarian steroids and peptides. In the male, release of FSH and LH under the control of the GnRH neurons results in secretion of testosterone and production of mature spermatozoa. The regulation of the hypothalamic-pituitary-ovarian (HPO) axis is extremely complex and involves feedback mechanisms. Moreover, different areas of the central nervous system can specifically modulate GnRH neurons. In this chapter we will discuss the regulation of the different components of the HPO axis.

The GnRH pulse generator

By analogy to the heart, the GnRH neurons are the pacemaker of the HPO axis. These neurons are located in the arcuate nucleus of the hypothalamus and secrete GnRH [1]. This decapeptide was discovered by Guillemin and Schally, who were awarded the Nobel Prize in 1977 for this milestone discovery [2]. The unique property of the GnRH neurons is that they secrete GnRH in pulses [3, 4]. GnRH pulsatility is an intrinsic property of these neurons, as demonstrated in animal experiments where explanted GnRH neurons maintain pulsatile secretion [5]. The axons of the GnRH neurons release pulses of GnRH into the pituitary portal circulation [6] (Fig. 7.1). There are only two portal circulations in the body, one in the liver and one involving the pituitary stalk and gland. A portal circulation is essential to allow sufficient amounts of GnRH to reach the pituitary gland. Without this system, the short half-life of GnRH [2–4] min and dilution in the systemic circulation would result in insufficient levels within the pituitary.

The pituitary portal vessels bring GnRH to the anterior pituitary where it can bind to its receptor located on the gonadotropes. These are the cells where LH and FSH are synthesized and released into the circulation. Importantly, since GnRH is released in a pulsatile fashion, LH and FSH are also released into the circulation in pulses [7]. Knobil *et al.* have demonstrated in a classic study that GnRH must be secreted in pulses in order to induce physiological LH and FSH secretion [8]. In this study involving rhesus monkeys with inactivated GnRH neurons, *continuous* (non-pulsatile) administration of exogenous GnRH inhibited FSH and LH release. In contrast, when exogenous GnRH was administered *in pulses* (i.e. in a physiological manner), LH and FSH secretion was restored. This indicates that optimal GnRH pulsatility is essential for a normal pituitary response and subsequent normal gonadal function. In clinical practice, as GnRH cannot be measured in the systemic circulation, LH pulsatility can be measured as a marker of GnRH activity. Indeed, it has been well demonstrated that LH pulsatility is driven by intrinsic GnRH pulsatility, thus acting as the pacemaker of the HPO axis [9].

Another unique and clinically relevant characteristic of the GnRH neurons is their origin. These neurons do not develop within the brain, but during fetal life migrate from the olfactory placodes to their final destination in the hypothalamus [10, 11]. Kallmann's

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

syndrome is defined by anosmia and hypogonadotropic hypogonadism [12]. The latter arises as a result of failure of migration of the GnRH neurons from the olfactory placodes to the hypothalamus. Concurrently there is failure of development of olfactory bulbs resulting in anosmia.

The anterior pituitary gland

The pituitary gland is a major element of the endocrine system [13]. The anterior part comprises different groups of cells regulating not only the gonadal axis but also the thyroid, adrenal, somatrope axes as well as prolactin secretion. Specifically, the gonadotropes synthesize and secrete LH and FSH under the influence of GnRH.

LH and FSH are glycoproteins composed of two subunits (α and β). Both share the α subunit. The β subunit differentiates between them. Interestingly, the GnRH pulse frequency can influence exactly which gonatropin is released [14]. A fast GnRH pulse frequency favours LH secretion, while a slow pulse frequency favours FSH secretion. Furthermore, this emphasizes the importance of optimal GnRH pulse frequency for precise control of gonadal function. This precise coordination is important as FSH and LH have distinct and separate roles at specific times of the menstrual cycle.

The menstrual cycle

Traditionally, for clinical ease, the first day of menstruation is used as the first day of the menstrual cycle, and the entire cycle lasts 28 days [15, 16]. The first 14 days are called the follicular or proliferative phase and the next 14 days, the luteal or secretory phase. In fact, the length of the cycle can vary from 25 to 35 days. However, of clinical relevance, it is the luteal phase duration that is fixed at 14 days, whereas the follicular phase duration can vary.

Under the influence of FSH, the follicular phase corresponds to the recruitment of a cohort of antral follicles, followed by selection of a dominant follicle that is destined to ovulate. The follicular phase is dominated by estradiol secretion from the growing follicle. The increasing estradiol level during the follicular phase will stimulate endometrial growth, which is why this phase is also described as the proliferative phase. Following this phase, a LH surge will induce ovulation. After ovulation, the follicle undergoes a series of changes giving rise to the corpus luteum, which is supported mainly by LH. This marks the

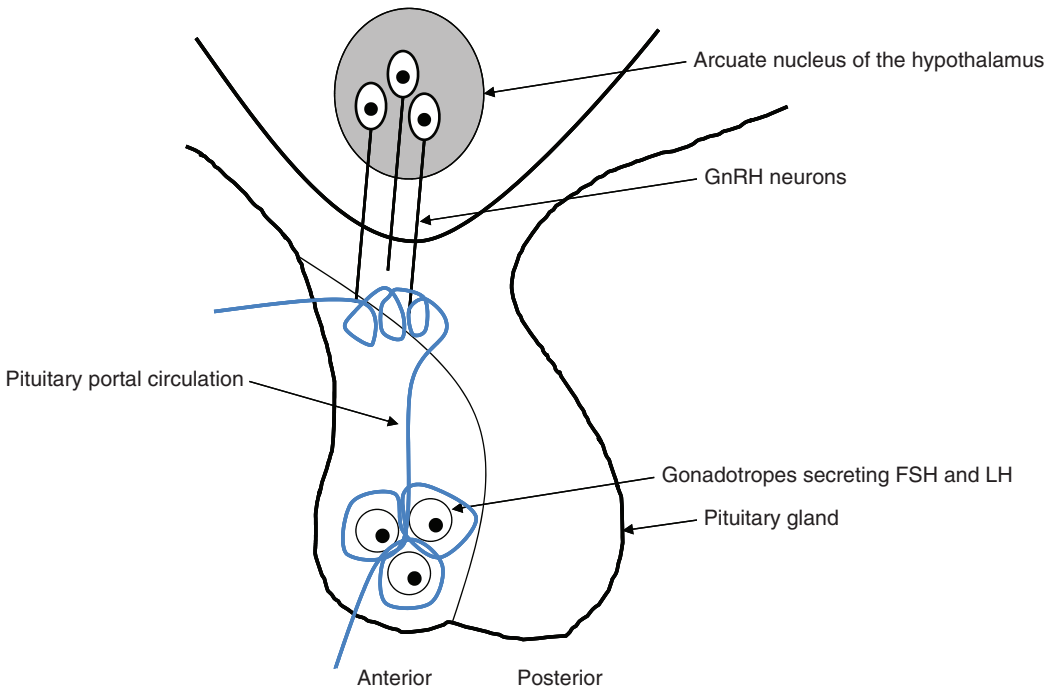

Figure 7.1 Axons of GnRH neurons release pulses of GnRH into the pituitary portal circulation.

beginning of the luteal phase. The dominant ovarian hormone at this time is progesterone along with concomitant estrogen secretion. Progesterone will induce secretory changes of the endometrium, hence this stage of the menstrual cycle is referred to as the secretory phase. Progesterone secretion prepares the uterus for implantation of an embryo. At the end of the luteal phase, ovarian hormone secretion declines. This triggers the release of digestive enzymes and prostaglandins, which act on the endometrium ultimately resulting in menstruation.

In order to comprehend how the gonadotropins regulate gonadal function, it is crucial to understand that the actual physiological beginning of the menstrual cycle occurs a few days before menstruation, induced by a small rise of FSH (signal FSH). The exact mechanism by which signal FSH occurs is not completely understood but may be related to a change in GnRH pulse frequency that favours FSH secretion. This rise of FSH will in fact, at that specific point in time, recruit the pool or cohort of antral follicles as described above. Physiologically, menstruation represents the end of the menstrual cycle in contrast to the conventional, clinical definition.

Modulation of the GnRH pulse generator and feedback mechanisms

In order to maintain the cyclicity of the menstrual cycle, it is essential to have exact synchronization between the different components of the HPO axis (hypothalamus, pituitary and the ovaries).

Control of GnRH pulses

As mentioned above, other neural centres can modulate pulsatile GnRH secretion and as such gonadotropin secretion downstream. Dopamine neurons located in the arcuate nucleus of the hypothalamus have been shown to suppress gonadotropin secretion via an inhibitory effect on the GnRH neurons [17]. Corticotropin-releasing hormone (CRH) and vasopressin (AVP) are two neuropeptides expressed in the paraventricular nucleus (PVN) of the hypothalamus, which centrally regulate the hypothalamic-pituitary-adrenal (HPA) axis, also known as the stress axis. CRH and AVP have been shown to inhibit the GnRH pulse generator and therefore mediate the inhibitory effect of stress on the reproductive axis [18, 19]. Noradrenaline, secreted from cells located in the locus coeruleus, is another important modulator of the GnRH neurons and is also involved in the negative effect of stress on the HPO axis [20].

Neuropeptide Y, synthesized in neurons of the arcuate nucleus, can stimulate pulsatile GnRH release [21]. Under specific conditions, neuropeptide Y (NPY) has also been shown to inhibit pulsatile GnRH secretion [22]. Since NPY can stimulate food intake and has been shown to be chronically elevated in different animal models of negative energy balance, it could be seen as one of the factors linking malnutrition and inhibition of reproduction [23]. Clinically the most extreme example is anorexia nervosa, where severe food restriction is associated with amenorrhea. Another neuropeptide, agouti-related peptide (AGRP), which is co-located in the same neurons as NPY and secreted in the same conditions, could also play a role in the inhibitory effect of malnutrition on ovarian function by decreasing the activity of the GnRH pulse generator [24].

Ghrelin is secreted from the stomach and has been shown to stimulate appetite through central activation of the NPY/AGRP neurons [25, 26]. Ghrelin is chronically elevated in patients with anorexia nervosa [27]. In a rhesus monkey model, administration of ghrelin has been shown to decrease LH pulse frequency and therefore could also be a component of the link between undernutrition and reproduction potentially through activation of the NPY/AGRP neurons [28].

Leptin is a peptide secreted by adipose tissue into the systemic circulation [29]. Leptin will then reach the hypothalamus where its main role is to decrease food intake. Moreover, leptin has been shown to be an important regulator of the reproductive axis. Leptin-deficient ob/ob mice not only have infertility from hypogonadotrophic hypogonadism, but also exhibit delayed puberty [30]. This indicates that leptin, as a sensor of energy stores, is a crucial factor linking nutrition and reproduction.

Kisspeptin is a newly discovered peptide that is also expressed in the arcuate nucleus of the hypothalamus [31]. It has been shown to stimulate GnRH release by direct action on the neurons. Indeed, GnRH neurons express the kisspeptin receptor. Moreover, kisspeptin may also act directly on pituitary gonadotropes to stimulate gonadotrophin release. Kisspeptin might play a key role in mediating gonadal steroid feedback to the hypothalamus. It could also be an important factor regulating pubertal development and may provide a link between nutritional status and fertility.

HPO feedback loops

Coordination between the different elements of the HPO axis is achieved by fascinating physiological mechanisms known as feedback loops. A feedback loop is a mechanism encountered in a variety of endocrine systems. An example of a negative feedback loop is when a pituitary hormone stimulates a target hormone such as the ovary. The hormone released by the ovary will then in turn inhibit the secretion of that pituitary hormone. An inhibitory feedback mechanism such as this will enable adjustment of hormonal secretion by the target organ. These feedback loops are essential for cross-communication between the ovaries and the other elements of the reproductive axis. The HPO axis is regulated by both negative and positive feedback loops as well as other peptides, which will be described below.

As described earlier, the FSH signal recruits a pool of follicles from which a dominant follicle will be selected. The growing follicle secretes increasing levels of estradiol that will have an inhibitory effect (*negative feedback*) at the hypothalamic and pituitary level, resulting in decreased LH and FSH release [32]. This will prevent another pool of follicles from being recruited. Then, at the end of the follicular phase, when estradiol reaches a certain threshold for a sustained period of time, it will exert a stimulatory *positive feedback* also at the hypothalamic and pituitary level [33]. This results in a surge of LH secretion accompanied by FSH release. This LH surge is the pivotal process by which ovulation occurs. It is quite striking that the same hormone can exert positive and negative feedback loops on the HPO axis. The exact mechanism controlling the positive feedback is currently not completely elucidated. Following ovulation, progesterone becomes the main hormone of the luteal phase and exerts a negative feedback on LH and FSH secretion.

Inhibins/activins/follistatin

Besides the negative and positive feedback loops described above, there are different peptides also involved in the fine regulation of the HPO axis. These include the inhibin family as well as the activins and follistatin. Inhibins are glycoproteins composed of two subunits (α and β) [34]. The β subunit differentiates between the inhibin A and inhibin B that have distinct functions. Inhibin B is synthesized in small follicles under FSH stimulation and in turn can exert negative feedback on FSH secretion. Inhibin B levels begin to increase rapidly after the FSH signal peaking at the beginning of the follicular phase and then decreasing at the end of the follicular phase. The negative feedback of inhibin B on FSH at the beginning of the follicular phase may play a role in the selection of a dominant follicle by preventing other follicles from being stimulated. There is a second increase of inhibin B occurring after the ovulatory LH surge. The physiological role of the second inhibin B increase is not completely understood. Studies in premenopausal women suggest that a decrease in inhibin B may be the earliest marker of the decline in follicle number across reproductive age. Inhibin A is secreted by the granulosa cells of growing follicles. Inhibin A levels only start rising at the end of the follicular phase and are maximal during the midluteal phase. The main role of inhibin A is to suppress FSH secretion during the luteal phase. Activins are a family of peptides secreted from the granulosa cells as well as the gonadotropes in the pituitary gland. They are involved in modulation of FSH secretion mostly by increasing its activity [35]. Follistatin is another peptide secreted from the granulosa cell whose main action is to decrease FSH activity partly by modulating activin [35].

Endogenous opiates

The endogenous opiates comprise a variety of peptides derived from precursors. Regarding the control of the reproductive axis, endorphins that are derived from proopiomelanocortin (POMC) play a major role. POMC is found in many different organs including the hypothalamus, where it is spliced into β-endorphin and α-melanocyte stimulating hormone (α-MSH). Beta-endorphin is a very important neuropeptide when discussing the regulation of the HPO axis [36]. Endorphin secretion varies according to the phases of the menstrual cycle. The highest levels are seen during the luteal phase, whereas the lowest levels are detected during menstruation. Progesterone can stimulate endorphin release and this effect can be augmented by estradiol [37]. It has been shown that endorphins can decrease the activity of the GnRH pulse generator and therefore decrease gonadotropin release [38]. During the luteal phase of the menstrual cycle, when the highest levels of endorphins and progesterone are secreted, there is a decline in the activity of the GnRH pulse generator and LH pulse frequency. Therefore, the inhibitory effect of progesterone on the GnRH pulse generator during the luteal phase is partially mediated by endorphin release.

Clinical implications: GnRH agonists and antagonists

Since GnRH has been shown to be the pacemaker of the reproductive axis, intense research has focused on ways to modify this decapeptide whose half-life lasts 2–4 minutes. The structure of GnRH is depicted in Figure 7.2. Its half-life is short as the peptide bonds at three different positions (5–6; 6–7; 9–10) can be cleaved, rendering it inactive. Modification at these crucial points of cleavage can alter the activity of the peptide. Agonists of the GnRH receptor (usually called GnRH agonists) can be obtained by substituting the amino acids at the sixth or tenth position. These modifications of the original molecule prevent its degradation. The clinical implication of the Knobil *et al.* study mentioned above which demonstrated that continuous administration of GnRH resulted in gonadotropin suppression has led to the use of GnRH agonists in women to suppress the HPO axis. Actually, the initial response after administration of a GnRH agonist is an increase in LH and FSH. This is known as the flare effect and corresponds to the release of the stock of pituitary gonadotropins. After this first effect, GnRH agonist will create a state of pseudomenopause, i.e. a hypogonadotropic hypogonadal state. This is known as down regulation of the pituitary. Therefore, GnRH agonists are often prescribed in situations where a suppressed HPO axis is needed, such as in patients with endometriosis and breast or prostate cancer. Additionally, GnRH agonists are a fundamental component of in vitro fertilization (IVF). By suppressing the HPO axis with a GnRH agonist, exogenous gonadotropins can be administered to stimulate the ovaries without causing an LH surge. If the agonist is not administered, a premature LH surge will occur and correct timing of egg collection is clinically difficult. During an IVF cycle, ovulation is induced by giving exogenous HCG (mimicking an LH surge) and egg collection is performed approximately 35 hours after the injection. The side effects of GnRH agonists may not be well tolerated and are comparable to the symptoms of menopause.

More recently substituting multiple amino acids of the GnRH structure has resulted in competitive GnRH antagonists. The most important difference with the GnRH agonists is the absence of the flare effect. GnRH antagonists are used in antagonist IVF protocols. During this protocol the ovarian stimulation usually starts on day 2 of the menstrual cycle. The GnRH antagonist is added on day 5 or according to the follicular size also to prevent a premature LH surge.

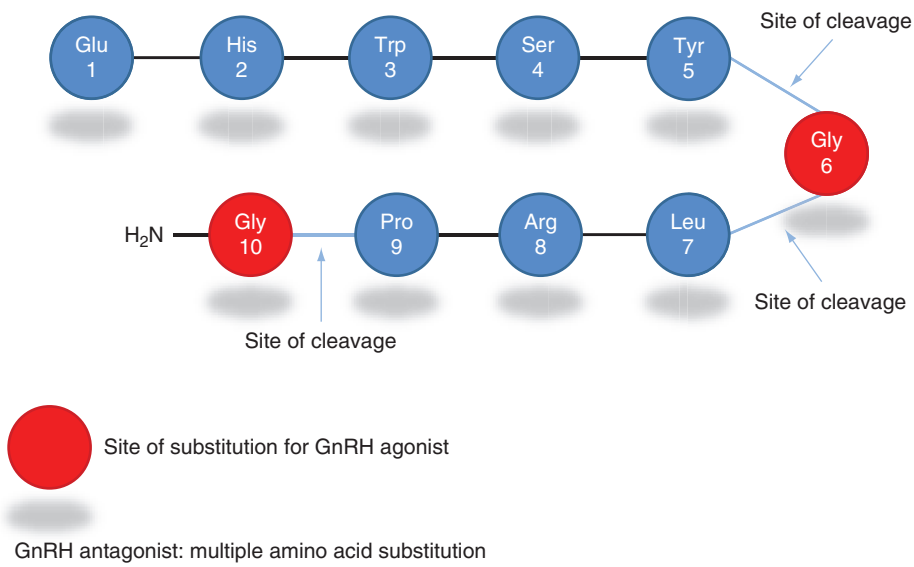

Figure 7.2 Structure of GnRH with its site of cleavage and the sites of amino acid substitutions for GnRH agonists.

As a result, an antagonist cycle is often termed a 'short' cycle. Aside from the reduction in treatment length, women usually do not experience the menopausal side effects that are associated with GnRH agonist cycles.

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Chapter 8

Reproductive endocrinology

Enda McVeigh

It is the intention of this chapter to provide a brief overview of reproductive endocrinology and how the mechanisms involved relate to reproductive medicine. It should be noted that the discipline of reproductive endocrinology is vast, and as such, it is simply beyond the scope of this chapter to provide a complete synthesis. Rather, this review will provide only a brief summary focusing particularly upon steroid hormones, menarche, amenorrhea and oligomenorrhea (including PCOS) and menopause.

Steroid hormones

Steroid hormones are predominantly synthesized in the gonads (testis and ovary), the adrenal glands, and during gestation, by the fetoplacental unit. Steroids act both on peripheral target tissues and the central nervous system (CNS). Gonadal steroids influence sexual differentiation of the genitalia and of the brain, determine secondary sexual characteristics during development and sexual maturation, contribute to the maintenance of their functional state in adulthood and control or modulate sexual behaviour. There are five major classes of steroid hormones: progestagens (progestational hormones), glucocorticoids (anti-stressing hormones), mineral corticoids (Na^+ uptake regulators), androgens (male sex hormones) and estrogens (female sex hormones).

Steroids are lipophilic, low molecular weight compounds derived from cholesterol (Fig. 8.1) which exhibits a cyclopentanophenanthrene ring structure that cannot be broken down in mammalian cells. Cholesterol contains 27 carbon atoms, all of which are derived from acetate. Cholesterol, and each of the steroid hormones, have four rings designated A, B, C and D. In steroid hormones, these rings are fused in a 'trans' orientation to form an overall planar structure. The conversion of C27 cholesterol to the 18-, 19- and 21-carbon steroid hormones involves the rate-limiting, irreversible cleavage of a 6-carbon residue from cholesterol, producing pregnenolone (C21) plus isocaproaldehyde. Steroids are extensively metabolized peripherally, notably in the liver, and in their target tissues, where conversion to an active form is sometimes required before they can elicit their biological responses. Steroid metabolism is therefore important not only for the production of these hormones, but also for the regulation of their cellular and physiological actions.

Steroid biosynthesis

The particular steroid hormone class synthesized by a given cell type depends upon its complement of peptide hormone receptors, the cell's response to peptide hormone stimulation and its genetically expressed complement of enzymes. Table 8.1 indicates which peptide hormone is responsible for stimulating the synthesis of which steroid hormone. The first reaction in converting cholesterol to C18, C19 and C21 steroids involves the cleavage of a 6-carbon group from cholesterol and is the principal committing, regulated and rate-limiting step for steroid biosynthesis. The enzyme system that catalyzes this cleavage reaction is known as P450-linked side chain cleaving enzyme (P450ssc), or 'desmolase', and is found within the mitochondria of steroid-producing cells, but not in significant quantities in other cell types.

Steroids of the adrenal cortex

The adrenal cortex is responsible for the production of three major classes of steroid hormones: glucocorticoids, which regulate carbohydrate metabolism; mineralocorticoids, which regulate the body levels of sodium and potassium; and androgens, whose actions are similar to that of steroids produced by the male

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gonads. Adrenal insufficiency is known as Addison disease, and in the absence of steroid hormone replacement therapy can rapidly cause death (in 1–2 weeks). The adrenal cortex is composed of three main tissue regions: zona glomerulosa, zona fasciculata and zona reticularis. Although the pathway to pregnenolone synthesis is the same in all zones of the cortex, the zones are histologically and enzymatically distinct, with the exact steroid hormone product dependent on the enzymes present in the cells of each zone.

Regulation of adrenal steroid synthesis

Adrenocorticotrophic hormone (ACTH), produced by the hypothalamus, regulates hormone production from the zona fasciculata and zona reticularis (Fig. 8.2). ACTH receptors in the plasma membrane activate adenylate cyclase by producing the second messenger, cAMP. The effect of ACTH on the production of cortisol is particularly important, with the result that a classic feedback loop is prominent in regulating the circulating levels of corticotrophin releasing hormone (CRH), ACTH and cortisol.

Table 8.1 Peptide hormones and associated steroid hormones

Peptide hormone	Steroid
Luteinizing hormone (LH)	Progesterone and Testosterone
Adrenocorticotrophic hormone (ACTH)	Cortisol
Follicle stimulating hormone	Estradiol
Angiotensin II/III	Aldosterone

Mineralocorticoid secretion from the zona glomerulosa is stimulated by an entirely different mechanism. Angiotensins II and III, derived from the action of the kidney protease renin on liver-derived angiotensinogen, stimulate zona glomerulosa cells by binding to a plasma membrane receptor coupled to phospholipase C enzyme. These events lead to increased P450ssc activity and increased production of aldosterone. In the kidney, aldosterone regulates sodium retention by stimulating gene expression of mRNA for the Na+/K+-ATPase responsible for the reaccumulation of sodium from the urine. Interplay between renin from the kidney and plasma angiotensinogen is important in regulating plasma aldosterone levels, sodium and potassium levels and ultimately blood pressure.

Disorders resulting from defects in steroid biosynthesis

A number of endocrine disorders can be attributed to specific enzyme defects. The inability to secrete normal levels of adrenal steroids may result in congenital adrenal hyperplasia (CAH) following hyperstimulation by ACTH (the negative steroid feedback controlling adrenal activity being lost). In the majority of cases, this syndrome is due to 21-hydroxylase deficiency and is associated with increased adrenal androgen secretion and partial virilization in girls. Less common adrenal enzyme deficiencies involve either 17-hydroxylase (with a possible increase in mineralocorticoid levels) or 18-hydroxylase (aldosterone may be deficient with normal levels of cortisol).

Gonadal steroid hormones

The two most important steroids produced by the gonads are testosterone and estradiol. These compounds are under tight biosynthetic control, with

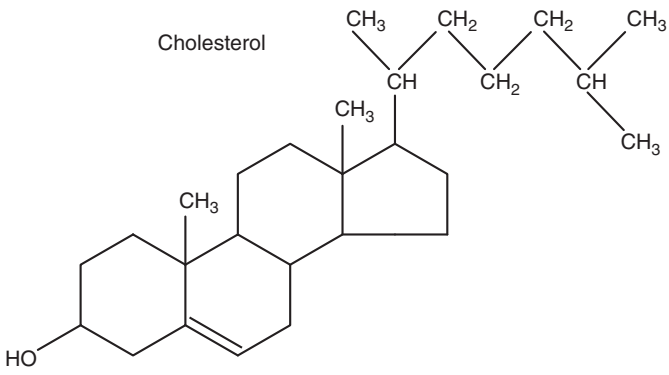

Figure 8.1 Structure of cholesterol.

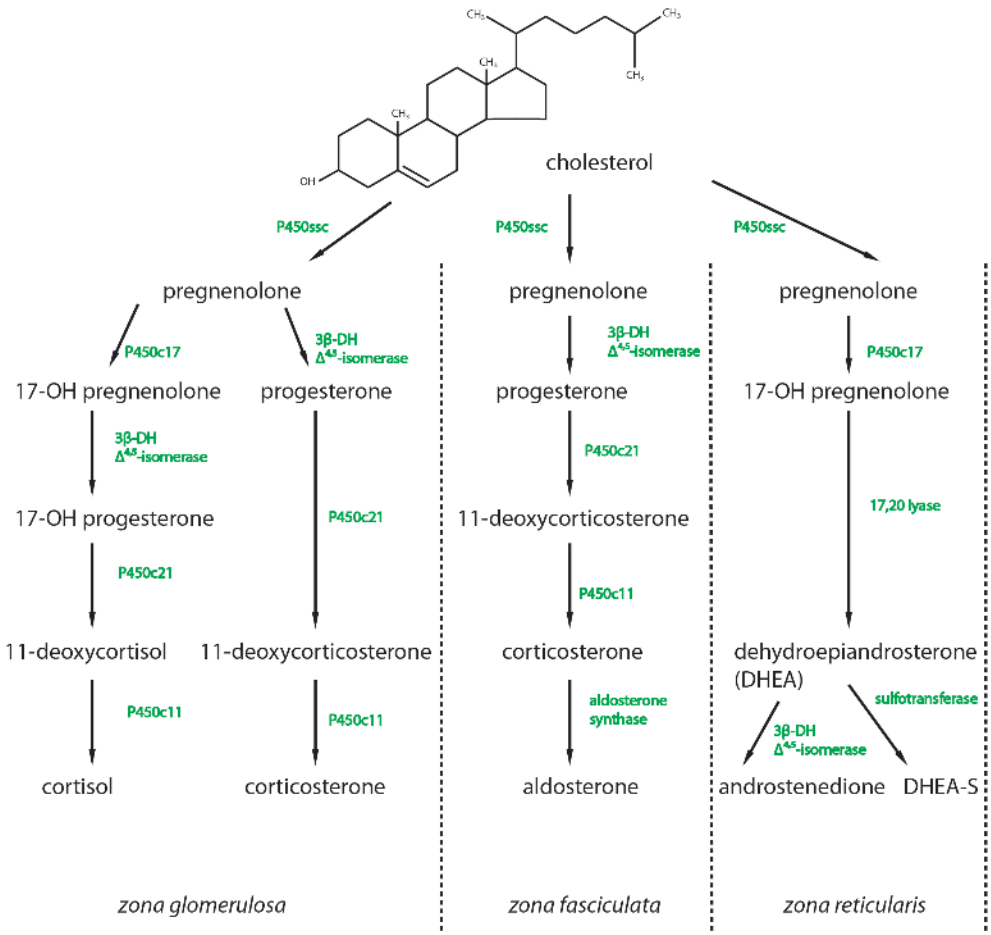

Figure 8.2 Synthesis of the various adrenal steroid hormones from cholesterol.

short and long negative feedback loops that regulate the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary, and gonadotropin releasing hormone (GnRH) by the hypothalamus. The biosynthetic pathway to sex hormones in male and female gonadal tissue includes the production of the androgens, androstenedione and dehydroepiandrosterone. Testes and ovaries contain an additional enzyme, 17-hydroxysteroid dehydrogenase, which enables androgens to be converted to testosterone. In males, LH binds to Leydig cells, stimulating production of the principal Leydig cell hormone, testosterone. Testosterone is secreted to the plasma and also carried to Sertoli cells by androgen-binding protein (ABP). In Sertoli cells, the Delta-4 double bond of testosterone is reduced, producing dihydrotestosterone. Testosterone and dihydrotestosterone are carried in the plasma, and delivered to target tissue, by a specific gonadal-steroid binding globulin (GBG). In a number of target tissues, testosterone can be converted to dihydrotestosterone (DHT). DHT is the most potent of the male steroid hormones, with an activity that is ten times that of testosterone. Because of its relatively lower potency, testosterone is sometimes considered to be a prohormone.

Steroid-binding proteins

Because of their lipophilic properties, free steroid molecules are only sparingly soluble in water. In biological fluids, they are found either in a conjugated form, i.e. linked to a hydrophilic moiety (e.g. as sulfate or glucuronide derivatives) or bound to proteins (non-covalent, reversible binding). In the plasma, unconjugated steroids are found mostly bound to carrier proteins.

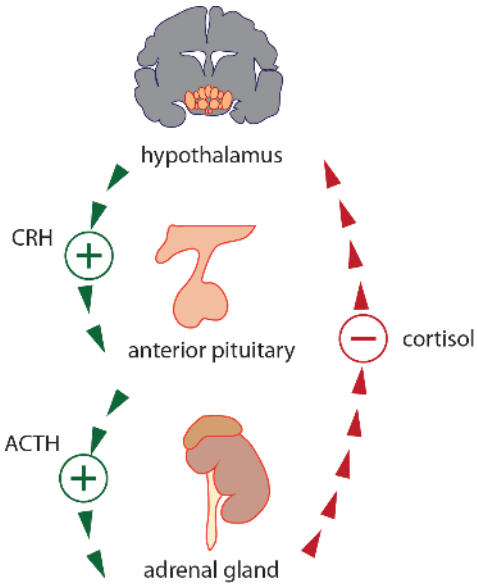

Figure 8.3 Feedback loop for the control of cortisol production.

Binding to plasma albumin accounts for 20–50% of the bound fraction and is rather unspecific, whereas binding to either corticosteroid-binding globulin (CBG) or the sex hormone-binding globulin (SHBG) is based upon more stringent stereospecific criteria. The free fraction (1–10% of total plasma concentration) is usually considered to represent the biologically active fraction. Apart from the two functions mentioned above, the major roles of plasma-binding proteins appear to be (a) to act as a 'buffer' or reservoir for active hormones and (b) to protect the hormone from peripheral metabolism (notably by liver enzymes) and increase the half-life of biologically active forms.

Menarche

Puberty marks the change from childhood to adolescence, and in girls is associated with the development of breasts, secondary sexual hair and the onset of menstruation. At the same time, there is a period of accelerated growth. The age at which these changes take place is variable, but it is abnormal for there to be no signs of secondary sexual development at the age of 14 years.

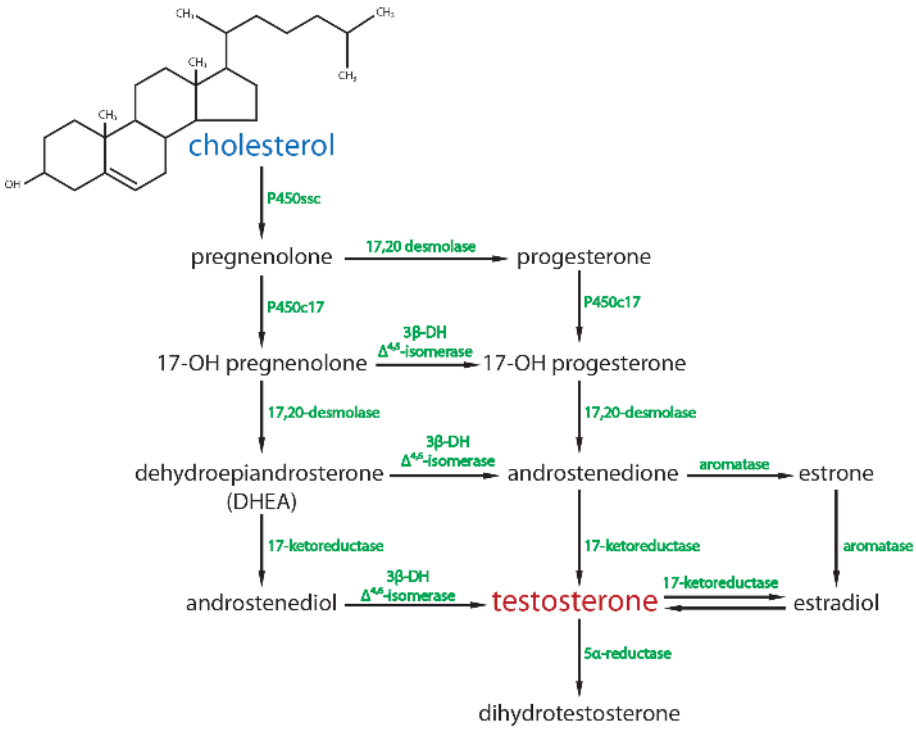

Figure 8.4 Gonadal steroid genesis.

The trigger for these changes to start is an increasing frequency and amplitude of gonadotrophin release. The ovaries are then stimulated to produce estrogen which acts upon breast tissue to promote growth. This usually begins at around the age of nine and takes about five years to be complete. Pubic hair is stimulated by the release of androgens from the ovaries and the adrenal glands. The age of menarche in girls appears to be decreasing, particularly in African American girls. Factors such as general health, nutrition (weight) and exercise all appear to have a role in the age of onset.

The hypothalamic-pituitary-gonadal axis

During fetal life, GnRH activity from the hypothalamus (which is present from around 20 weeks) is suppressed by steroid production from the fetoplacental unit. The ovaries therefore have only minimal estrogen output. During infancy, there is an increase in GnRH activity in boys aged 6 months, and in girls from around 12 months. This leads to an increase in production of testosterone in boys and estradiol in girls. At this early age the feedback mechanism to the pituitary is immature. As this feedback mechanism matures over a few months in childhood, the levels of FSH and LH decrease. In girls, this leads to the lowest levels of FSH and LH at around 4 years old.

At around 6 years of age, girls exhibit an increase in the amplitude and frequency of GnRH production from the hypothalamus. This is then associated with the onset of diurnal rhythms of FSH, LH and steroids. Puberty progresses with an increase in nocturnal amplitude of LH and a gradual change to an adult pattern of 90-minute pulses. This is similar in both boys and girls. In boys, this diurnal rhythm results in peak testosterone in the early morning leading to erections; boys enter puberty about six months later than girls but are fertile earlier with spermaturia from 6ml testicular volume. In girls the diurnal rhythm results in an estrogen rise later during the night as this estrogen requires aromatization. This results in peak values mid-morning. Subsequent ovulatory cycles develop about two years after menarche. FSH pulsatility shows no diurnal variation at any stage, with only a slight increase in amplitude but not frequency as puberty progresses.

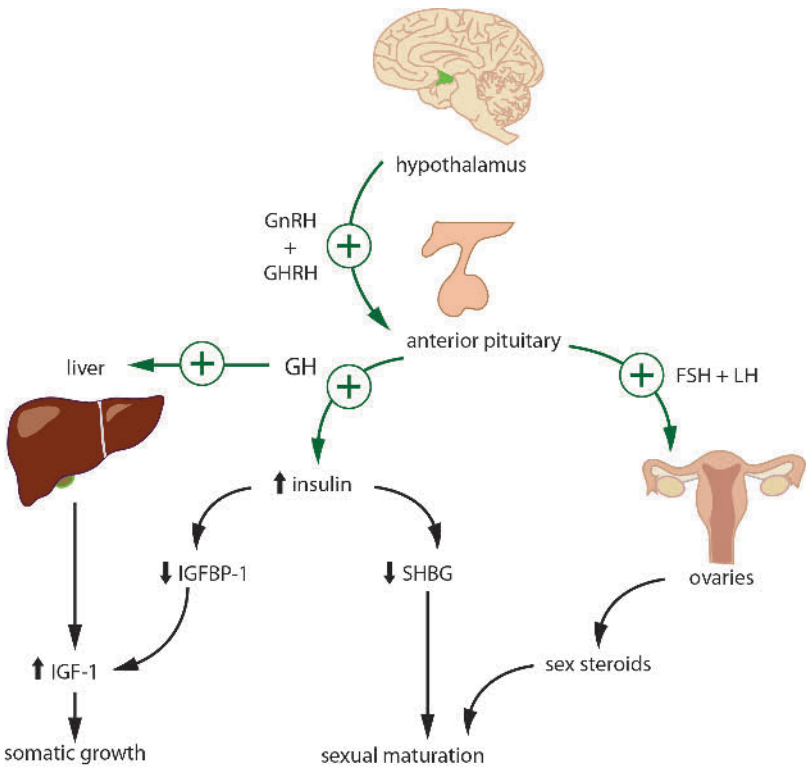

Figure 8.5 Puberty and the hypothalamic-pituitary-ovarian axis.

Table 8.2 Marshall and Tanner staging criteria

Stage	Breast	Pubic Hair
I	Pre-adolescent, elevation of papilla only	No pubic hair
Ii	Breast bud – elevation of breast papilla as small mound; enlargement of areolar diameter	Spare growth of long downy hair along labia
Iii	Further enlargement but no separation of contours	Hair coarser, darker and more curled; over mons
Iv	Projection of areola and papilla to form secondary mound above the level of breast	Adult type hair but no spread to thigh
V	Mature, areola recessed to general contour of breast	Adult, with horizontal upper boarder and spread to thigh

Stages of puberty

In girls, breast and pubic hair development is described in five stages following the classification by Marshall and Tanner; see Table 8.2.

- Sexual characteristics appear in 95% of girls between 8.5 and 13 years
- 10–12.5 years Breast development (average age breast stage 2 = 11.2 years)
- Pubic hair usually six months after breasts appear, although before breasts in one-third of population
- 1 year later adolescent growth spurt
- Menarche: 12–15 years, as growth spurt wanes, average age 13 years

Precocious puberty

Precocious onset of puberty is defined as when puberty occurs younger than 2 standard deviations (SD) before the average age; < 8 years old in females and < 9 years in males. The incidence of precocious puberty is approximately 1 in every 5000–10 000 individuals.

Causes of precocious puberty

There are several causes of precocious puberty: idiopathic (family history, overweight/obese accounts for 74% girls and 60% boys – abnormal levels of TGFα may stimulate GnRH secretion), McCune Albright syndrome (café au lait spots and polyostotic fibrous dysplasia), tumours of the adrenal or ovary producing steroids, Peutz-Jeghers syndrome, cerebral tumours such as intra-cranial lesions (tumours, hydrocephalus, CNS malformations, irradiation, trauma: tumours are suspected if the subject is < 3 years old), and the ingestion of exogenous estrogens.

The management of precocious puberty is to initially investigate and exclude tumours. GnRH agonists (e.g. depot) can be used to suppress the hypothalamic-pituitary-gonadal axis. It is also important to assess bone age (from the wrist) to predict potential epiphyseal fusion and whether the patient may benefit from receiving growth hormone, but this will depend on the age.

Delayed puberty

Delayed onset of puberty is defined as when puberty occurs at an age older than 2 standard deviations (SD) beyond the average age (> 13.4 years old in females, and > 14 years in males). A detailed history should be taken including aspects of general health. In girls, the history should include the age at which breast and pubic hair development started and if the girl had a growth spurt or still appears to be growing. Any chronic illness may lead to constitutional delay in puberty. Examination should include accurate measurement of height, and in the female case, breast and pubic hair development. An internal examination should not be performed on girls. Investigations may include: measurement of gonadotrophins – FSH and LH and estrogen, karyotyping, ultrasound scan of the pelvis to confirm the presence of uterus and ovaries, and possibly an X-ray to determine bone age.

Causes of delayed puberty

The most common condition seen by pediatric endocrinologists is 'constitutional delay of growth and puberty'. This is usually associated with a positive family history, short stature, delayed epiphyseal maturation and a relatively short upper body. The height prognosis may be appropriate for parental centiles, although in severe cases, the upper body may remain short. Treatment may be for psychological reasons, with low-dose ethinylestradiol. This condition usually resolves with the onset of breast development and a growth spurt. Other factors are given in Table 8.3. Cases of hypogonadotrophic hyogonadism may be difficult to distinguish with constitutional delay. Sometimes GnRH tests can be helpful but results may be unreliable. The management of delay in puberty will

Table 8.3 Factors associated with delayed onset of puberty

Condition	Additional Information
Malabsorption	e.g. coeliac disease, inflammatory bowel disease
Underweight	e.g. dieting/anorexia nervosa, over-exercise
Other chronic diseases	e.g. malignancy, asthma, β thalassaemia major
Gonadal failure	Hypergonadotrophic hypogonadism
Turner's syndrome	–
Post-malignancy	Chemotherapy, local radiotherapy or surgical removal
Polyglandular autoimmune syndromes	–
Gonadotrophin deficiency	–
Congenital hypogonadotrophic hypogonadism (± anosmia)	A number of diagnoses possible
Idiopathic	–
Kallmann's syndrome (X-linked)	Impaired migration of GnRH neurons, anosmia, disturbance of colour vision, dyskinesis
Prader-Willi syndrome (aut. dom., chromosome 15)	Obesity, muscle hypotonia, mental retardation, short stature, small hands/feet, cryptorchidism, HH
Mutations in pathway for GnRH secretion and action	e.g. KAL, DAX1, GnRH receptor
Hypothalamic/ pituitary lesions	Tumours, post radiotherapy. Rare inactivating mutations of genes encoding LH, FSH or their receptors

follow the diagnosis but is usually accomplished with low-dose oestradiol (2 mg slowly rising) or pulsatile GnRH or Gn (FSH+LH) therapy.

The menstrual cycle

Normally, ovulation occurs once a month in the fertile age range between menarche and menopause, although anovulation generally occurs at the extremes of reproductive life. A cycle is regarded as normal if the duration is 24–35 days. The time between menstruation and ovulation is termed the 'follicular phase' and between ovulation and the next menstruation, the 'luteal phase'. Ovulation itself is the release of a mature fertilizable oocyte from the dominant follicle, the culmination of an integrated, synchronized interplay of hormones from three principal sources: the anterior hypothalamus (gonadotrophin releasing hormone, GnRH), the anterior pituitary (FHS and LH) and the ovaries (17 estradiol, E2; progesterone, Prog). In addition, fine tuning is provided by inhibin, activin, follistatin and various other growth factors.

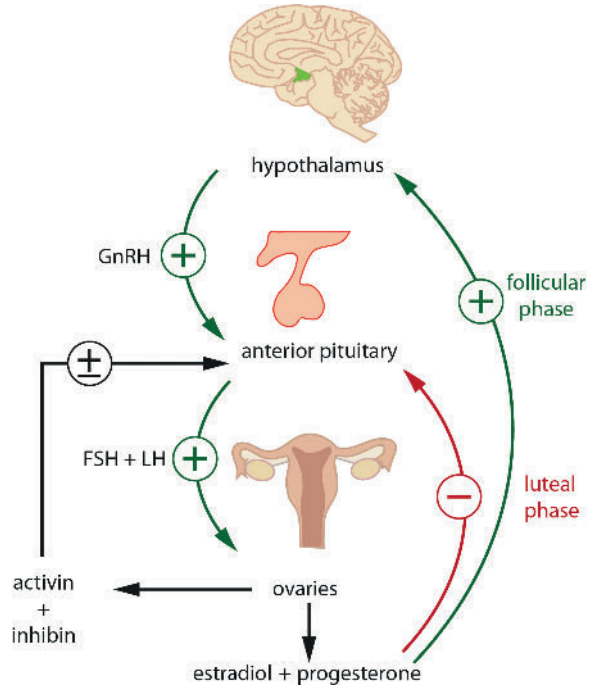

Figure 8.6 The hypothalamic-pituitary-ovarian (HPO) axis.

Ovulation is achieved through the synchronization of the timing of release and quantity of the various hormones involved which change throughout the cycle as a result of feedback mechanisms (discussed below).

GnRH

GnRH is secreted in a pulsatile fashion from nerve endings in the hypothalamus into the portal vessels, running a short course to the anterior pituitary where it induces the synthesis and release of FSH and LH. GnRH is undetectable in the peripheral circulation but its pulsatile release, about once every hour, can be estimated from LH pulses. Both the frequency and amplitude of GnRH pulses vary greatly throughout the ovulatory cycle, and are much less frequent, but of greater amplitude, in the luteal phase compared with the follicular phase. The pattern of GnRH release is influenced by feedback mechanisms on the hypothalamus and dictate the pattern of release of FSH and LH.

FSH

Immediately preceding menstruation, FSH levels start to rise as corpus luteum function fades and reach a peak around day three of menstruation. The FSH stimulated growth of antral follicles, granulosa cell proliferation and differentiation and aromatase action produce rising concentrations of estradiol and inhibin B which exert a negative feedback mechanism. Other than a temporary increase at the time of the mid-cycle LH surge, FSH remains low until the end of the luteal phase. FSH has several roles and promotes granulosa cell proliferation and differentiation, antral follicle development, oestrogen production, induction of LH receptors on the dominant follicle and inhibin synthesis.

LH

LH is the main promotor of the constant production of androgens, the substrate of ovarian steroid hormones, from theca cells. Concentrations of LH are uneventfully low throughout the ovulatory cycle except for one tumultuous rise at mid-cycle to 10–20 times the resting levels. This surge lasts for 36–48 hours and is brought about by a dramatic effect of rapidly rising estradiol levels which reach a certain concentration and initiate a switch from negative to positive feedback. The preovulatory surge has several functions: triggering of ovulation and follicular rupture, disruption of the cumulus-oocyte complex, induction of the resumption of oocyte meiotic maturation and the luteinization of granulosa cells.

Estradiol

17-β estradiol, the most important estrogen, is produced by granulosa cells under the influence of FSH which promotes the action of the enzyme aromatase in converting basic androgens to estrogen. The key functions of estradiol are: endometrial development, triggering of the LH surge at mid-cycle, suppression of FSH concentrations to aid selection of the dominant follicle and the prevention of multi-follicular development in the mid- to late follicular phase.

Estradiol concentrations rise rapidly following menstruation to reach a peak in the late follicular phase and induce the LH surge. A slight decrease following ovulation is revived by production from the corpus luteum which falls sharply immediately before menstruation.

Progesterone

The main function of progesterone is to stimulate a secretory endometrium containing multiple tortuous glands receptive to a fertilized embryo, allowing it to implant. It also stimulates the expression of genes needed for implantation. As progesterone is produced by luteinized granulosa cells, its concentration only rises to significant amounts following ovulation and declines rapidly with the demise of the corpus luteum before menstruation. Progesterone reaches peak levels in the mid-luteal phase. A blood sample for progesterone at this time, e.g. day 21 of a 28-day cycle or day 28 of a 35-day cycle, is used to confirm ovulation.

The ovary

During the reproductive life span, the ovary is a very dramatically changing organ. The inner, medullary or stromal section is made up of connective tissue inundated by small capillaries and adrenergic nerves. The cortex contains an enormous number of oocyte-containing follicles ranging from approximately 300 000 at menarche to 1500 at menopause. There is a constant state of flux in the various stages of development of the follicles from primordial (an oocyte with a single layer of granulosa cells around it), through primary and secondary stages with increasing numbers of layers of granulosa cells, antral stages containing follicular fluid, to a fully fledged, preovulatory follicle. A corpus luteum can be seen in the luteal phase of the cycle and the picture is completed by the presence of corpora albicans (remnants of degenerate corpora lutea).

Although much of this changing picture in terms of follicular development is dependent on the stage of the ovulatory cycle, there is a constant, non-FSH dependent progression in development of primordial to potentially ovulatory follicles being available at the start of the ovulatory cycle, a process that may take about ten weeks.

Follicular development

One follicle a month (i.e. about 400 in a reproductive life span) will be selected for ovulation. The remainder, 99.9% of those that started life in the ovary, become atretic. The earliest stage of follicular selection starts some ten weeks before the cycle for which it is intended. This is a constant non-FSH dependent step-up leading to the growth of primordial cells to several surviving, potentially ovulatory follicles

2–5 mm in diameter. Sensitivity to FSH then comes into play to select the follicle for further growth, granulosa cell differentiation and multiplication. As estrogen and inhibin are produced by growing follicles, FSH concentrations are reduced, making it less available. The follicle most sensitive to FSH becomes dominant and the rest fade into atresia. The dominant follicle is the main producer of estradiol due to aromatase action stimulated by FSH. The dominant follicle also develops LH receptors in the late follicular phase in preparation for the LH surge and impending ovulation.

Amenorrhea and oligomenorrhea

Amenorrhea is the absence of menstruation for at least six months. Primary amenorrhea is defined if a menstrual period has never occurred and secondary amenorrhea after at least one period. Oligomenorrhea is the occurrence of menstruation less than once in 35 days – six months, or less than nine times in one year. Physiological amenorrhea is an acceptable diagnosis before the onset of menarche (unless this has not occurred before the age of 17 years), following the menopause (if this occurs after the age of 40 years), during pregnancy or during lactation. All other causes of amenorrhea and oligomenorrhea are listed according to a modified World Health Organization (WHO) classification, composed of five groups: hypothalamic-pituitary failure (WHO Group 1), hypothalamic-pituitary dysfunction (Group 2), ovarian failure (Group 3), hyperprolactinaemia (Group 4) and outflow tract defect (Group 5). The classification of oligo/amenorrhea, common causes and hormonal profiles are summarized in Table 8.4.

Patients classified as WHO Group 2 may present with oligo- or amenorrhea and represent the vast majority of these types of disorders that are seen. Characterized by normal FSH and estradiol concentrations, almost all these cases are associated with polycystic ovary syndrome (PCOS). PCOS is characterized by oligo- or amenorrhea, clinical and/or biochemical hyper-androgenism (hirsutism, persistent acne, raised testosterone concentrations) and a typical polycystic appearance of the ovary on ultrasound examination. Two or more of these three diagnostic points are enough to confirm the diagnosis, assuming other causes of hyper-androgenism have been ruled out. Many women with PCOS are overweight or obese, hyper-insulinaemic and infertile. The basic aetiology is unknown but it is thought to be associated with an overproduction of androgens by the ovaries which, in the majority of these women, seems to be genetic in origin.

Table 8.4 Causes of amenorrhea and oligomenorrhea

Condition	Further information
Hypothalamic-pituitary failure	Due to hypogonadotrophic-hypogonadism, in which concentrations of both FSH and LH are so low as to be unable to stimulate follicle development or ovarian steroidogenesis. Amenorrhea, anovulation and hypo-estrogenism are the consequences. There are several possible causes of this condition.
Weight-related amenorrhea	Not uncommon. Due to loss of weight during severe dieting or frank anorexia nervosa.
Exercise-related amenorrhea	Caused by very strenuous exercise such as marathon running, other athletic pursuits and not uncommon in ballet dancers.
Stress-related	Includes even moderate stress, e.g. moving house, before examinations, long journeys involving time shifts, etc.
Kallmann's syndrome	Hypothalamic amenorrhea associated with anosmia (loss of the sense of smell)
Debilitating systemic diseases	–
Craniopharyngioma	–
Idiopathic	Probably the commonest 'cause' of primary amenorrhea
Surgical	e.g. Hypophysectomy
Radiotherapy	For pituitary or surrounding area tumours
Sheehan's syndrome	Hypogonadotrophic hypogonadism and hypopituitarism following severe post-partum hemorrhage
Hypothalamic-pituitary dysfunction	–

Ovarian failure

Ovarian failure is responsible for about 10% of women with secondary amenorrhea before the age of 40 years (premature menopause), but may also be a cause of primary amenorrhea. This form of amenorrhea is characterized by hypo-estrogenism and high concentrations of FSH (often > 25IU/L). The ovaries in this condition are unable to respond to endogenous or exogenous FSH

as they are either completely devoid of oocytes, or have a severely depleted reserve of oocytes. Possible causes include secondary amenorrhea – premature menopause, familial/genetic factors, auto-immune abnormality, iatrogenic factors – chemotherapy or direct radiation of the ovaries, pelvic surgery, debilitating systemic disease, infection (e.g. mumps), idiopathic factors, primary amenorrhea, chromosomal abnormalities – gonadal dysgenesis (e.g. Turner's syndrome, 45XO, characterized by its typical physical features of short stature, cubitus valgus, webbed neck and 'streak' ovaries and sometimes associated with aortic stenosis, intersexuality and hermaphroditism, hyper-prolactinaemia (caused by either either oligo- or amenorrhea, infertility and often, but not always, galactorrhea. Conversely, galactorrhea is not always accompanied by hyper-prolactinaemia).

Hyper-prolactinaemia is most commonly caused by pituitary adenoma (prolactinoma) – almost invariably benign tumours that secrete prolactin. According to their size, prolactinomas may be termed macroadenomas (> 10 mm in diameter) or microadenomas (< 10 mm) when visualized by MRI or CT scan. When large, these adenomata may impinge on the optic chiasma, inducing a bi-temporal hemianopia. Another factor causing hyper-prolactinaemia is hypothyroidism. Thyroid stimulating hormone (TSH) releasing hormone is also thought to be a prolactin releasing hormone. As TSH concentrations (and, by inference, TSH releasing hormone) are often elevated in hypothyroid conditions, these may often be associated with hyper-prolactinaemia sufficient to cause oligo- or amenorrhea. Many drugs used in psychiatric conditions, such as sedatives or anti-emetics, suppress the hypothalamic secretion of dopamine. As dopamine is thought to be a prolactin-inhibiting factor, these medications can often induce hyper-prolactinaemia and consequential oligo- or amenorrhea. Oral contraceptives and other estrogen-containing medications may also induce a mild hyper-prolactinaemia, often associated with galactorrhea. Stress, particularly if prolonged, may cause hyper-prolactinaemia sufficient to induce oligo- or amenorrhea.

Unlike the aforementioned causes of amenorrhea, outflow tract defects are not usually associated with anovulation but with a mechanical defect preventing menstruation. Possible causes include: imperforate hymen, congenital absence of the uterus, transverse vaginal septum, severe intra-uterine adhesions/endometrial damage (Asherman's syndrome) or cervical stenosis.

The treatment of oligo- and amenorrhea depends not only upon aetiology but also on the purpose of the treatment, basically, whether there is a problem of infertility or not. Except for women with outflow tract defects, the rest may be assumed to have oligo- or anovulation, and if pregnancy is desired, then ovulation induction will be needed.

Medical treatment of amenorrhea and oligomenorrhea

Hypothalamic-pituitary failure

For ovulation induction, gonadotrophin treatment, which must contain both FSH and LH, is very effective. If the pituitary is intact, pulsatile GnRH therapy is equally effective. If the cause of the amenorrhea is a low body weight, it is highly recommended that the patient gain weight before embarking on ovulation induction therapy in order to avoid associated complications of pregnancy. If pregnancy is not wanted, hormone replacement therapy with estrogens and progesterone, similar to that used in the menopause, is called for, in order to avoid osteoporosis or any other possible effects of prolonged hypo-estrogenism.

Hypothalamic-pituitary dysfunction

For women diagnosed as having PCOS and suffering infertility, a range of possible treatments for ovulation including weight loss, clomiphene citrate, and low-dose gonadotrophin therapy is possible. For those who have PCOS, but for whom infertility is not the presenting complaint, several options are open and may be tailored to the individual case. Weight loss is an essential first step for the overweight or obese. A loss of just 5% or more of body weight may be enough to restore ovulation and menstruation. For those suffering from symptoms of hyper-androgenism (hirsutism, acne, alopecia), a combination of the anti-androgen cyproterone acetate (CPA, and ethinyl estradiol (EE) is probably the most widely used treatment.

Ovarian failure

For patients desiring pregnancy, ovum donation is the only successful option. Otherwise, hormone replacement therapy, as for menopausal patients, is recommended.

Hyper-prolactinaemia

When hyper-prolactinaemia and oligo- or amenorrhea are associated with medication, the benefits and disadvantages of reducing dosage or withdrawing medication must be carefully weighed. Hypothyroidism as a cause should be treated with the appropriate medication for correction of thyroid function rather than with specific prolactin-lowering agents. All other cases of hyper-prolactinaemia associated with ovulatory dysfunction and oligo/amenorrhea, whether idiopathic or from a pituitary tumour, require treatment. Neurosurgical treatment for the treatment of hyper-prolactinaemia is very rarely required today. For both micro- and macroprolactinomas, prolactin-lowering drugs are safer, more efficient and often capable of causing tumour shrinkage without recourse to surgery. Surgery should be reserved only for the very rare case that is completely resistant to medication, for non-secreting pituitary adenomas, or para-sellar tumours, and in those who have severe visual disturbances which fail to improve with medication. For all the rest, prolactin-lowering medication (dopamine agonists, Bromocriptine or Carbergoline) will serve the purpose adequately.

Outflow tract defects

Imperforated hymen and transverse vaginal septa are treated with relatively simple surgical techniques to restore the integrity of the outflow tract. Imperforate hymen is probably the most frequent obstructive anomaly of the female genital tract, but estimates of its frequency vary from 1 case per 1000 population to 1 case per 10 000 population.

Menopause

The term menopause is derived from the Greek 'menos' (month) and 'pauses' (cessation), but the term has come to be used to describe the climacteric, which again is derived from the Greek word 'klimakter' (rung of ladder). The average age of the menopause has not changed, but life expectancy has improved to the extent that in the UK, women can expect to spend about one-third of their lives in a menopausal state. Menopause is usually defined retrospectively, one year after the last menstrual period (average age 51). The term 'climacteric' refers to the 'climb' to the menopause (average age of 45–47 years, lasting 4 years on average, though can last up to 10 years). Early menopause is diagnosed if the patient is < 45 years, or premature ovarian failure if < 40 years.

The number of primordial follicles that a female has shrinks throughout life with replacement. In a newborn, there are an estimated 2 million primordial follicles, 3–400 000 at puberty, a few thousand at an age of 40+ years and few or no oocytes post-menopause. The number of ovarian follicles available to mature each cycle is depleted (3–400 cycles on average) as women get older. As one oocyte ovulates, approximately 1000 become atretic through apoptosis. There are two critical landmarks in the ovarian failure process – the first is a marked decline in fertility (no cycle dysfunction) and the second occurs when the menstrual cycle changes noticeably, with a shortened follicular phase and luteal dysfunction.

The effect of the reduced pool of follicles for stimulation is that the estrogen levels start to fall. Initially there is a 'compensated failure'. This is then associated with an increase in the production of FSH and a decrease in the level of inhibin produced by the follicles. Early follicular Inhibin B and FSH appear to be predictive of ovarian reserve / response to gonadotrophin stimulation. FSH level will however vary in the climacteric patient with a non-linear increase and currently we are awaiting more population data on inhibin B. Due to the lack of more population data on inhibin B, the standard test remains FSH alone. 'Decompensated failure' occurs when the follicle pool is very low. The FSH rises further (ten- to twenty-fold); LH rises threefold (shorter half-life). Estrogen levels drop due to reduction in follicle number and qualitative effect on granulosa cell aging. There is a permanent cessation of progesterone production which can lead to endometrial proliferation and hyperplasia.

Other hormonal changes

Adrenal and ovarian androgens (testosterone and androstenedione) decline. Some testosterone is still, however, produced by theca cells. Ovarian androstenedione production drops by half in menopause so that the majority is from the adrenals (1:4 ratio). Sex hormone binding globulin (SHBG) decreases due to reduction in ovarian estradiol. The main source of post-menopausal estrogen is estrone, produced mainly in peripheral adipose tissue and the post-menopausal ovary by aromatization of adrenal androstenedione. The amount of estrone produced is related to body weight and age. Glucocorticoid administration in post-menopausal patients will suppress estrogen

production, confirming that it is from an adrenal production site. Insulin resistance also rises after the menopause. This change results in increased central adiposity (android rather than gynecoid shape) and a decreased lean body mass.

The characteristic symptoms of the menopause are shown in Table 8.5. Although the hot flush may characteristically start over the face or neck area, it involves the whole body and is often followed by intense sweating, and then by shivering. Hot flushes occur in 70% of Caucasian and Afro-Carribbean women, but are less common in Japanese and Chinese women, possibly due to a cultural mechanism or by a diet rich in isoflavone. Hot flushes are not present in Turner's or lifelong hypothalamic amenorrhea patients. Obese women are partially protected, probably due to their high estrone production and lower SHBG levels. It is thought that the mechanism is such that estrogen induces hypothalamic opiod activity, and this loss of activity can then lead to thermo-dysregulation, mediated by noradrenaline. Estrogen also increases alpha2 adrenergic activity, leading to flushing, and hence the rationale for clonidine therapy.

CNS systems

Estrogen and progesterone receptors are co-located in the CNS, in the hypothalamus, amygdala, pre-optic area, hippocampus and the cerebellum. In these areas they mediate genomic effects, e.g. limbic system functions subserving emotion and behaviour. Estrogen has a direct effect on 5HT and noradrenaline receptors. It increases the rate of degradation of MAO, thus increasing levels of 5H. Estrogen also displaces tryptophan from albumin, providing more 5HT substrate as well as enhancing the transport of 5HT.

The depression that is seen at the menopause is partly due to serotonin and noradrenaline deficit. Estrogen increases the levels of these neurotransmitters. The effect of estrogen supplements, in the form of HRT at the menopause, on cognitive function is unclear. Some trials indicate estrogen improves function as indicated by memory and attention improvements. Current evidence from randomized controlled trials remains inadequate.

Urogenital factors

Women may experience a number of symptoms arising from the urogenital system around the menopause (Table 8.6). Most of these symptoms are a result of atrophy of vaginal and urethral epithelium (estrogen receptors) with loss of rugations and stenosis. Reduced cell maturation leads to a decreased number of superficial cells. There is a disturbance of the vaginal flora (decreased lactobacilli, increased faecal flora) and a resultant increase in vaginal pH. In the peri-urethral connective tissue there is also a decreased amount of collagen.

Skeletal system

Bone mass reaches a peak towards the end of the woman's third decade (Fig. 8.7). It then remains relatively stable until the menopause, after which the loss is lifelong. Seventy per cent of women over the age of 80 will have measurable osteoporosis. It is estimated that there are some 60 000 hip fractures, 50 000 Colles fractures, and 40 000 clinical apparent vertebral fractures a year in the UK. Factors that can affect the bone mass include genetics/race and diet/calcium during adolescence. Factors affecting bone loss include premature menopause, amenorrhea, exercise/diet/weight, smoking/alcohol/caffeine or the use of corticosteroids. Risk factors affecting the chances of fracture include: low bone mass, low body weight, current cigarette

Table 8.5. Acute and intermediate / late symptoms of the menopause

Acute	Intermediate / late
Hot flushes 70%	Dyspareunia
Night sweats 70%	Loss of libido
Insomnia	Urethral syndrome
Anxiety/irritability	Vaginal atrophy
Memory loss	–
Poor concentration	–
Mood changes	–

Table 8.6. Possible urogenital symptoms associated with the menopause

Vaginal symptoms	Urinary symptoms
Vaginal dryness, irritation, discharge	Recurrent urinary tract infections
Vulvo-vaginal pruritus, pain	Urinary frequency, urgency
Dyspareunia	Dysuria, voiding difficulties
Postcoital bleeding	Urinary incontinence
Prolapse	
Anorgasmia	

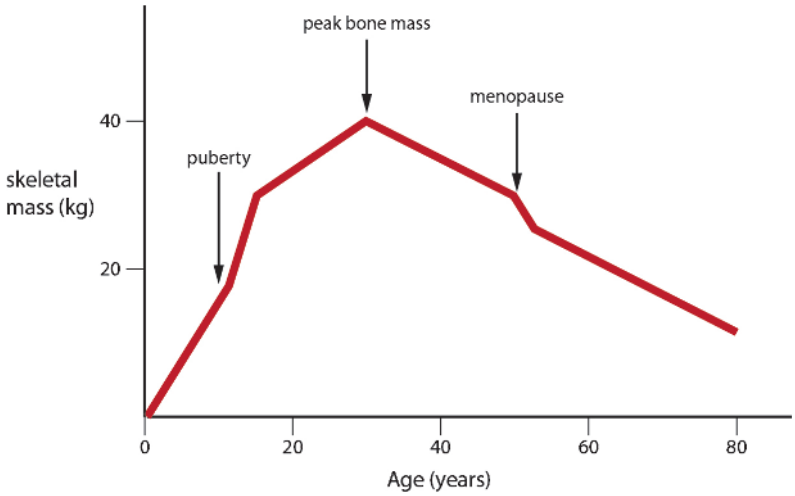

Figure 8.7 Changes in skeletal mass with women's age.

smoking, personal or family history of fracture, risk factors for falls, confusion disorders, medications (sedative hypnotics, alcohol), neuromuscular disease and environmental factors.

Cardiovascular risk factors

Coronary heart disease (CHD) is uncommon among premenopausal women, particularly if they do not smoke. There is however a rapid increase in risk following the menopause, and cardiovascular disease is now a leading cause of death among postmenopausal women. The mechanism that provides premenopausal women with CHD protection is not clear; however, it is known that estrogen has a number of protective effects including: reduction in myocardial ischaemia, nitric oxide mediated vascular dilatation, inhibition of platelet aggregation, increased HDL and decreased LDL, reduction in insulin resistance, antioxidant effects on endothelial cells.

The reduction of increased risk of CHD in women on HRT after the menopause was addressed in two large studies: Women Health Initiative (WHI) trial [1] and Million Women Study [2]. The WHI trial was set up with the primary aim to test whether the postmenopausal use of HRT protected women from CHD. The study was a randomized controlled trial which enrolled more than 16 000 American women. Women were randomized to take HRT in the form of 0.625 mg of conjugated equine estrogen and 2.5 mg of medroxyprogesterone acetate daily, or placebo. After 5 years of followup the women on HRT were found to have: a higher incidence of breast cancer, a higher incidence of myocardial infarction, stroke and pulmonary embolus, lower CHD risk in 50–59 year old women and a reduced incidence of hip fractures and colorectal cancers.

The Million Women Study was a UK-based study that collected data from women attending for breast screening as part of the NHS breast screening programme. One million women were followed between May 1996 and March 2001. The women were between 50 and 64 years of age. Half of the women used HRT at some point, with half of those taking the combined hormone medication. Results of this study showed: combined estrogen / progestagen HRT was associated with a twofold increase in breast cancer when compared with non-users; and that estrogen-only HRT represented a 30% increased risk of breast cancer. Looking at a ten-year period, the risk of breast cancer was four times greater in those taking a combined HRT than an estrogen-only preparation.

UK committee on safety of medicine and HRT

Following publication of these two significant studies, the UK Committee on Safety of Medicine issued advice to prescribers of HRT. This advice can be summarized as follows. For short-term (e.g. 2–3 years) use of HRT for the relief of menopausal symptoms, the benefits outweigh the risk for most women. Longer term use of HRT is licensed for the prevention of osteoporosis. However, patients should be aware of the increased incidence of some conditions with

long-term HRT use and of alternative options for the prevention of osteoporosis. The decision to use HRT should be discussed with each woman on an individual basis, taking into account her history, risk factors and personal preferences. Individual risk and benefits should be regularly reappraised (e.g. at least annually) while using HRT, and also HRT should not be used for the prevention of CHD.

Acknowledgement

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Chapter 9

Reproductive immunology

Ian Sargent

Introduction

Despite many advances in assisted reproduction technology, success rates are still disappointingly low. While the use of techniques such as intracytoplasmic sperm injection has meant that most couples are now able to achieve fertilization, many apparently normal embryos fail to implant. One possible cause for this could be that the mother mounts an abnormal immune response to the implanting embryo. The immune paradox of pregnancy was first recognized by the Nobel Prize-winning immunologist Sir Peter Medawar [1]. He realized from his pioneering work on tissue transplantation that, as half of the fetus's genes are inherited from the father, the mother's immune system should recognize the fetus as foreign and reject it as she would a foreign kidney graft or heart transplant. As this clearly does not happen in normal pregnancy, he speculated about the mechanisms which might be involved in preventing this rejection. Failure of these protective mechanisms to different extents could potentially lead to the poor placentation seen in preeclampsia, miscarriage and in the worst cases, implantation failure.

Medawar put forward three proposals as to how the fetus might avoid maternal immune recognition:

(1) There is an anatomical separation of the fetal antigens and maternal immune system such that they never come in contact to elicit a response.
(2) The antigens expressed by the fetus are in some way 'immature' so that they cannot stimulate immune responses in the same way as antigens expressed on adult tissue.
(3) The maternal immune system is somehow suppressed during pregnancy.

These ideas have provided a useful framework for exploring the mechanisms of the feto-maternal immune relationship and will be discussed in this chapter to illustrate our current understanding of the field.

Is there anatomical separation of the fetus and mother?

It should be emphasized that the mother's immune system has no direct contact with the fetus. Rather it is the placenta that forms the interface with maternal tissues. There are two distinct placental interfaces which are important at different stages of pregnancy.

Interface 1

The first interface is that formed initially between the trophectoderm of the implanting embryo and later the developing placenta in the first trimester of pregnancy, and the overlying maternal decidualized endometrium (*decidua basalis*). The placenta is composed of 'root-like' structures called chorionic villi. The tips of some of these villi serve to anchor the placenta to the decidua. Specialized placental cells called extravillous cytotrophoblast invade from the tips of the anchoring villi into the decidual stroma (interstitial cytotrophoblast) or into the lumen of the decidual spiral arteries (endovascular cytotrophoblast) which supply maternal blood to the placenta (Fig. 9.1). The endovascular cytotrophoblasts strip away the endothelium of the spiral arteries and destroy the muscle layer, opening out the arteries to convert them from high-resistance/low-flow vessels carrying an estimated 45 ml blood per minute to low-resistance/high-flow vessels carrying 750 ml blood at term. This is thought to be a crucial mechanism to increase the blood flow to the placenta to sustain the fetus as it grows and protect the

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

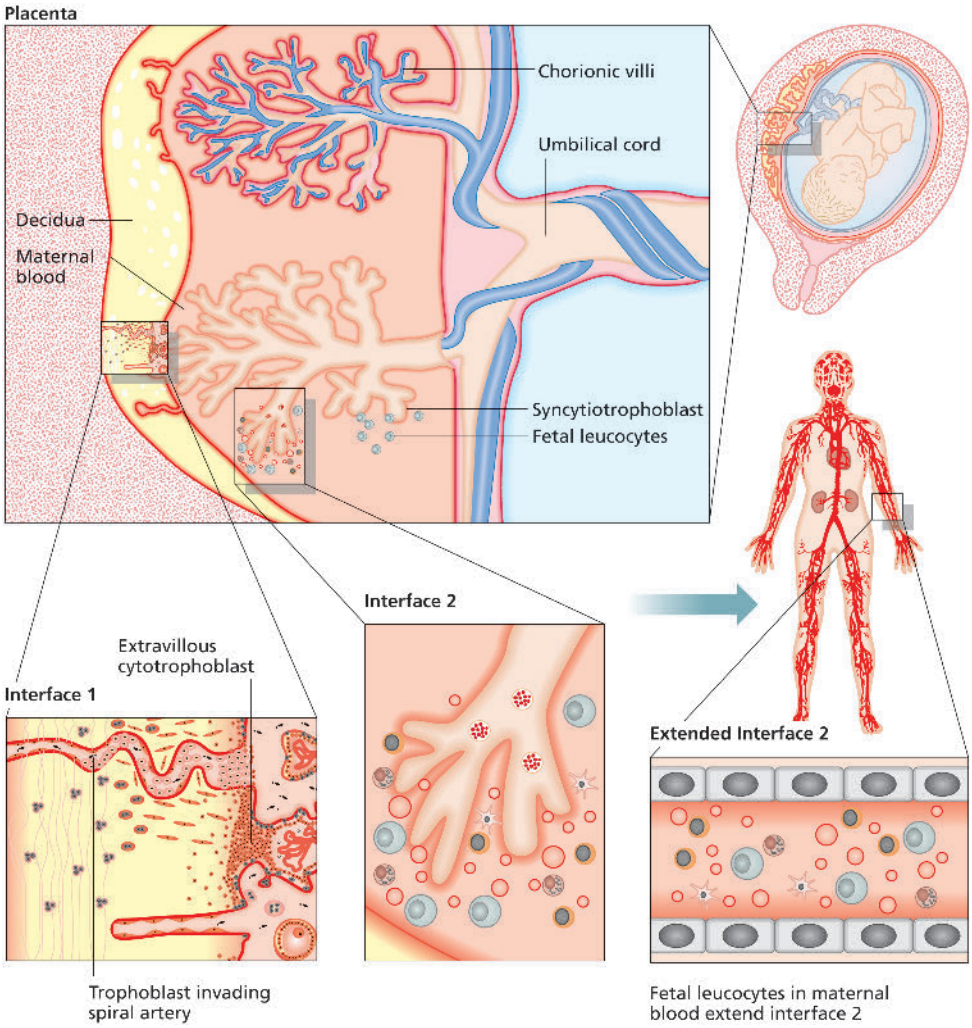

Figure 9.1 The two main immunological interfaces of pregnancy.

chorionic villi from potential damage from high blood pressure in the intervillous space.

Interface 2

The second interface is between the syncytiotrophoblast (the epithelium which covers the surface of the chorionic villi) and the maternal blood, and is established towards the end of the first trimester of pregnancy (Fig. 9.1). This interface is more important in the second half of pregnancy as the placenta grows and comprises a large surface area over which the exchange of gases and nutrients occurs to sustain the developing fetus. This interface is extended throughout the mother's circulation during pregnancy in two ways. First, there is shedding of cellular and microvesicular syncytiotrophoblast debris and cell-free DNA and mRNA from the surface of the placenta into the mother's blood throughout pregnancy [2], and second there are hemorrhages of fetal blood across the placenta. Thus, fetal red blood cells and leucocytes can be found in the maternal circulation as early as the first trimester where they can interact with maternal immune cells [3].

It is clear from the above that there is no anatomical separation of placental trophoblast and maternal cells per se. However, the key question is whether the trophoblast that comes in contact with maternal immune cells has the ability to elicit an immune response.

Cells of the innate and adaptive immune systems

Immune cells fall into two main groups: those of the innate and adaptive immune systems. The main cell types in the innate immune system are (1) granulocytes which phagocytose foreign material and destroy it, (2) macrophages (monocytes in the blood) which phagocytose infectious agents, destroy them and present them to T cells, (3) dendritic cells which are potent antigen-presenting cells, and (4) natural killer (NK) cells which destroy tumours and virally infected cells (especially those which lack major histocompatibility (MHC) antigens).

The adaptive immune system comprises B cells, which produce antibodies, and T cells of which there are four main types: (1) T helper 1 (Th1) cells which interact with monocytes and macrophages to help them destroy intracellular pathogens, (2) T helper 2 (Th2) cells which interact with B cells to help them divide, differentiate and make antibody, (3) T cytotoxic cells (Tc) which destroy foreign cells or host cells infected with virus or intracellular pathogens, and (4) regulatory T cells (Treg) which are a specialized sub-population of T cells that suppress activation of the immune system and are involved in transplantation tolerance.

In early pregnancy up to 40% of the cells in the maternal decidua (interface 1) are immune cells [4]. Approximately 10% of these are T cells (including regulatory T cells – see below) while there are very few B cells. About 20% of cells are macrophages and 2% are dendritic cells. However, the most predominant population of cells (up to 70%) are uterine natural killer (NK) cells. These are a specialized population of NK cells which differ from classical NK cells in the blood in that they do not express the immunoglobulin Fc receptor CD16 and highly express the adhesion molecule CD56, compared to the low expression seen on the majority of circulating NK cells (Table 9.1). Their function is also very different in that they have a much lower killing activity than peripheral blood NK cells but produce cytokines, chemokines and angiogenic factors. They are found in close association with the invasive extravillous cytotrophoblast, suggesting that they play an important role in feto-maternal immune interactions.

Table 9.1 Comparison of peripheral blood and uterine NK cells properties

Phenotypic markers	Blood CD56dim NK cells	Uterine CD56bright NK cells
CD56	+	++++
CD16	++	–
NK activity	High	Low
Cytokine production	–	++++

At interface 2, the syncytiotrophoblast is bathed in maternal blood and is therefore in direct contact with the full complement of peripheral blood immune cells (including T cells, B cells, NK cells, monocytes, granulocytes and dendritic cells).

Do the antigens expressed by the trophoblast differ from those expressed by adult tissues?

Medawar predicted that antigens expressed by the fetus (i.e. trophoblast) may differ in some way from those expressed by normal adult tissues and thereby evade maternal immune recognition.

The antigens involved in graft rejection responses are the molecules of the MHC. In the human there are two types of MHC antigen – Class I and Class II MHC.

Class I MHC

There are two major groups of class I MHC antigens: 'classical' class I molecules (HLA-A, HLA-B and HLA-C) and 'non-classical' class I molecules (HLA-E, HLA-F and HLA-G).

The classical molecules are constitutively expressed on most nucleated cells. They are highly polymorphic, with over 500 HLA-A, over 900 HLA-B and over 300 HLA-C alleles being described.

In contrast, the non-classical molecules exhibit a limited polymorphism and their tissue expression is also limited. HLA-E and HLA-F are expressed on a variety of adult and fetal tissues, while HLA-G is predominantly restricted to the invasive extravillous cytotrophoblast tissue of the placenta [5]. The function of Class I molecules is to present endogenous peptides derived from within the cell cytoplasm (including viral proteins) to CD8-positive cytotoxic T cells (Tc), which are then able to kill the infected or foreign cell.

Class II MHC

The MHC class II region encodes more than 30 proteins, including HLA-DR, HLA-DQ and HLA-DP. These antigens are also highly polymorphic (with more than 800 alleles having been described) and are expressed on specialized antigen-presenting cells (APCs) including monocytes, macrophages, B cells

Table 9.2 Trophoblast major histocompatibility antigen Class I expression

Tissue	HLA-A	HLA-B	HLA-C	HLA-E	HLA-F	HLA-G
Adult and fetal cells						
Invasive extravillous cytotrophoblast (interface 1)						
Syncytiotrophoblast (interface 2)						

and dendritic cells. APCs internalize foreign antigens (predominantly bacterial), either by phagocytosis or by receptor-mediated endocytosis. They then present peptides derived from these antigens via a class II MHC molecule on their cell membrane to CD4-positive T helper cells (Th). A Th cell response is therefore generated that eventually leads to the production of antibodies against the same peptide antigen.

The key question therefore is, do trophoblast cells express MHC antigens?

Trophoblast MHC expression

All types of trophoblast are negative for Class II MHC antigens. The syncytiotrophoblast is also negative for membrane-bound class I antigens, although it has been reported that a soluble form of HLA-G (see below) may be expressed. In contrast, the invasive extravillous cytotrophoblast has a unique pattern of Class I MHC expression, unlike that of any other cell in the body (Table 9.2). It does not express the classical HLA-A and HLA-B antigens, which are the principal stimulators of graft rejection. However, it does express HLA-C which, although less stimulatory, can still cause graft rejection. It also expresses the non-classical class I MHC antigens, HLA-E, HLA-F and HLA-G. As mentioned above, HLA-G is almost uniquely expressed in the extravillous cytotrophoblast, suggesting that it plays a key role in feto-maternal immune interactions. It displays a low degree of polymorphism but has a large variety of molecular structures as a result of alternative splicing. Four membrane-bound (HLA-G1, HLA-G2, HLA-G3, HLA-G4) and three secreted soluble (HLA-G5, HLA-G6, HLA-G7) isoforms have been described [5]. Soluble forms can also be generated by proteolytic cleavage of membrane anchored molecules.

Interaction of trophoblast MHC with maternal immune cells

The unique pattern of MHC expression shown by the extravillous cytotrophoblast is thought to be central to feto-maternal immune interactions. Two types of immune cell in the decidua are able to respond to MHC antigens – NK and T cells.

Uterine NK cells

The principal immune interactions in the decidua appear to be between trophoblast HLA-G, HLA-E and HLA-C and uterine NK cells rather than with T cells. However, these do not lead as would be expected to the destruction of the trophoblast; on the contrary they facilitate trophoblast invasion and placental development. This occurs through the interaction of the MHC antigens with NK cell receptors, leading to the inhibition of dangerous NK cell responses and the activation of beneficial ones. HLA-E binds to the inhibitory C-type lectin family receptor CD94/NKG2A which causes suppression of NK cell cytotoxicity, protecting the extravillous cytotrophoblast from NK cell attack [6], as does the binding of HLA-G to the Leukocyte Immunoglobulin (Ig)-like receptor LILRB1 (also known as Ig-like transcript – ILT1). Conversely, the binding of HLA-G and HLA-C respectively to the activating Killer Immunoglobulin-like Receptors (KIRs) KIR2DL4 and KIR2DS4 expressed on uNK cells stimulates the production of cytokines (e.g. IFNγ, IL-10, TGFb1, IL-6, IL-8, TNFα), chemokines such as IL-8 and IP-10 and also angiogenic factors such as VEGF and PLGF [7]. Together, these promote and regulate trophoblast invasion and angiogenesis in the decidua.

Changes in trophoblast MHC expression in pregnancy failure

If the expression of HLA-C, HLA-G, HLA-E and HLA-F by trophoblast is essential for successful pregnancy, then it might be expected that their expression is altered in cases of implantation failure, recurrent miscarriage and preeclampsia. HLA-G is expressed by human embryos from the two cell stage onwards and soluble HLA-G can be detected in the culture supernatants of IVF embryos. There is some evidence of a

positive correlation between HLA-G expression and embryo quality. This has led to the suggestion that soluble HLA-G could be used as a non-invasive marker for selecting IVF embryos with the greatest implantation potential. The ability to select the best embryos for transfer is becoming increasingly important with the move to single embryo transfer to reduce the number of multiple pregnancies after IVF treatment. However, the published data are conflicting and it is as yet unclear how useful measuring soluble HLA-G will be [8].

Several studies have reported decreased levels of HLA-G mRNA and protein expression on extravillous cytotrophoblast in unexplained recurrent miscarriage and preeclampsia which would reduce their immunoregulatory potential with uNK and decidual T cells. Lower levels of soluble HLA-G have also been reported in the maternal circulation in miscarriage and preeclampsia, although the functional significance of this is less clear. As yet, nothing is known about trophoblast expression of HLA-E and HLA-F in these conditions.

Trophoblast HLA-C and uNK cell KIR receptor interactions

The interaction between trophoblast HLA-C and uNK cell KIRs is of special interest as both systems are extremely polymorphic and the nature of the interaction depends on which genotypes are expressed. KIR haplotypes fall into two groups, A and B, the latter distinguished by having additional activating receptors. Possible maternal KIR genotypes are therefore AA (no activating KIR) or AB/BB (presence of one or more activating KIRs).Thus the secretion of beneficial cytokines, chemokines and angiogenic factors is enhanced by ligation of an activating haplotype B KIR and reduced by ligation of an inhibitory haplotype A receptor [9]. HLA-C haplotypes also fall into two main groups, C1 and C2, with HLA-C2 interacting with both inhibitory and activating KIRs more strongly than HLA-C1. Thus, fetal HLA-C2 on the trophoblast interacting with KIR BB haplotype uNK cells would lead to strong stimulation of the activating receptors and hence would be the best combination for promoting placentation. Conversely, uNK cells from KIR AA mothers confronted with HLA-C2 trophoblast would lead to strong stimulation of inhibitory receptors which would lead to poor placentation or pregnancy failure. This is indeed what is observed in preeclampsia and recurrent miscarriage and may explain the partner specificity which is well recognized in these conditions.

Decidual T cells

The absence of HLA-A and HLA-B expression on trophoblast means that the two most potent stimulators of T-cell-mediated rejection are missing at the maternofetal interface. However, HLA-C is also polymorphic and able to stimulate graft rejection responses. Furthermore, it has been shown that there is an increase in the percentage of activated T cells in the decidua of pregnancies with an HLA-C mismatched fetus. This raises the question as to how decidual T cells are prevented from destroying invasive extravillous cytotrophoblast cells. This is thought to be mediated through induction of regulatory T cells in the decidua. Their depletion leads to abortion in mice [10], and in the human their numbers are increased in the decidua in HLA-C mismatched, but not matched, pregnancies, suggesting they play a key role [11]. There is an increase in the numbers of circulating regulatory T cells (Tregs) in the late follicular phase of the menstrual cycle, with a further increase in pregnancy, peaking in the second trimester and declining towards term [12]. Higher numbers of Tregs are found in the decidua compared to the peripheral blood. This increase may be driven by trophoblast HLA-G which has been shown to induce suppressive activity of CD4+ Th cells [13]. Another candidate is indoleamine 2,3 dioxygenase (IDO), an important immune regulator. IDO works by catabolizing tryptophan in the microenvironment, thereby starving T cells of this essential amino acid, causing them to differentiate into Tregs. In the absence of IDO, T cells are reprogrammed to acquire a Th17 phenotype, with more proinflammatory activity. Treatment of pregnant mice with IDO inhibitor leads to the rejection of the fetuses [10].

Several studies have reported that the number of Tregs in both the peripheral blood and decidua are reduced in preeclampsia compared to normal pregnant women [12]. Consistent with this is the finding of lower levels of IDO in preeclampsia placentas which could lead to diminished Treg activity [14]. Furthermore, it has been reported that there is a bias towards Th17 cells over Tregs in women with preeclampsia. Reduced levels of Tregs have also been reported in the decidua and peripheral blood of women undergoing spontaneous abortion as well as reduced FOXP3 mRNA (a transcription factor characteristic of Tregs) in the endometrial tissue of women with primary infertility.

Is the mother's immune system suppressed during pregnancy?

The third potential mechanism for the survival of the fetus suggested by Medawar was that the mother becomes immunologically inert during pregnancy. Clearly there cannot be a blanket immune suppression, as pregnant women would be susceptible to all kinds of infections, and this is not the case. However, there are more subtle changes to the maternal immune system that occur which can be manifested both locally in the uterus and in the peripheral circulation.

Th1/Th2 changes in pregnancy

When a CD4-positive T helper is presented with an antigen by an antigen-presenting cell, it can differentiate in one of two ways depending on the cytokine environment to which it is exposed. IFN-γ and IL-12 enhance so-called Th1 development, leading to the activation of macrophages and CD8-positive T cells and thereby promoting cell-mediated immunity (Fig. 9.2a). Alternatively, IL-4 promotes differentiation into Th2 cells which stimulate B cells to produce antibody, promoting humoral immunity. In normal individuals, responses may go in either direction, allowing them to mount both cell-mediated (Th1) and antibody-mediated (Th2) responses. However, in pregnancy this equilibrium is altered because the placenta produces Th2-promoting cytokines, such as IL-4 and IL-10, as well as progesterone, which inhibit Th1 responses (Fig. 9.2b). Thus, the bias in normal pregnancy is towards Th2 immunity, with suppression of cell-mediated immunity (in particular suppression of IFN-γ production), which

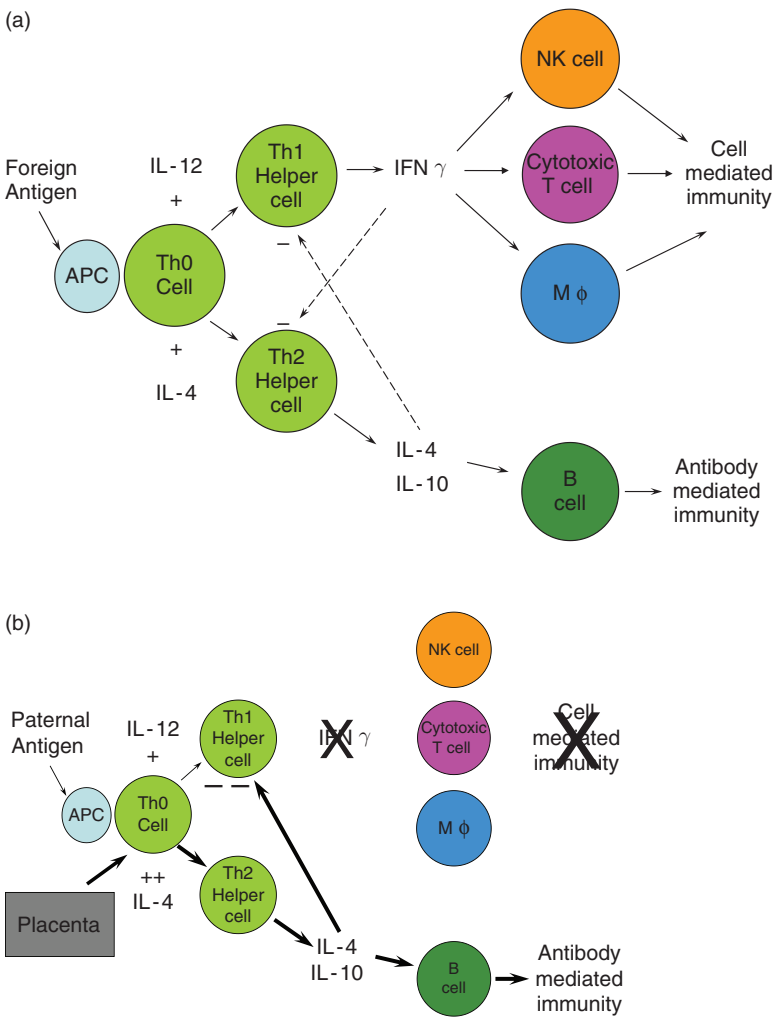

Figure 9.2 Th1/Th2 cytokine balance in pregnancy. (a) Th1/Th2 balance in a non-pregnant woman. (b) Th1/Th2 balance in pregnancy.

will help to protect the fetus. This still leaves the pregnant woman with her antibody-mediated immunity intact and thus she is still able to fight infection. Clinical evidence for a shift towards Th2 and away from Th1 responses in normal pregnancy includes: (1) temporary remission of rheumatoid arthritis (Th1-mediated) during pregnancy, (2) exacerbation of diseases caused by intracellular pathogens (for example herpes and malaria) during pregnancy, and (3) worsening of systemic lupus erythematosus (Th2-mediated) during pregnancy.

Antibody production in pregnancy

The ability of the mother to mount a normal antibody response in pregnancy presents a potential problem if she is able to make antibodies to paternal antigens expressed by the fetus. Although the extravillous cytotrophoblast and syncytiotrophoblast do not appear to stimulate classic acquired (T-cell and B-cell) immune responses because of their pattern of MHC expression, fetal leucocytes that enter the maternal circulation express a full complement of HLA-A, HLA-B and HLA-C antigens and therefore have the potential to stimulate the production of anti-fetal antibodies. These are found in 15% of first pregnancies and 60% of subsequent pregnancies by the same father. However, these antibodies do not seem to harm the pregnancy in any way. The problem arises as one of the major functions of the placenta is to transport maternal antibodies to the fetal circulation [15]. In this process, maternal IgG antibodies in the mother's blood bind to Fc receptors on the surface of the syncytiotrophoblast and are actively transported into the chorionic villus where they enter the fetal circulation. These antibodies provide immediate protection for the fetus against infections to which the mother has been exposed. Only IgG antibodies and not IgM, IgE, IgA or IgD are transported in this way. However, the potentially harmful anti-paternal HLA antibodies are filtered out by the placenta before they reach the fetal blood. In this situation the antibodies are still transported across the syncytiotrophoblast, but they bind to paternal HLA antigens expressed on the macrophages and endothelium of the chorionic villus. Here the immune complexes formed are cleared by the macrophages and complement regulatory factors (e.g. decay accelerating factor – DAF) inhibit complement activation. Thus none of the harmful antibodies enter the fetal circulation.

Hemolytic disease of the newborn

One situation where this mechanism breaks down is in hemolytic disease of the newborn, or rhesus disease. This occurs when a mother makes IgG antibodies to antigens present on the surface of her fetus's red blood cells which have crossed the placenta and entered her bloodstream. These antibodies are then transferred back across the placenta into the fetal circulation and cause the destruction of fetal erythrocytes [16]. The antigen most common in such a condition is rhesus D (RhD). During her first pregnancy with an RhD-positive baby, an RhD-negative mother is only exposed to small numbers of fetal RhD-positive erythrocytes by hemorrhages of fetal blood across the placenta. However, at delivery, separation of the placenta allows significantly more fetal blood to enter the mother's circulation. These fetal red blood cells are recognized by the maternal immune system, leading to the production of RhD-specific memory B cells postpartum. The first incompatible child is therefore usually unaffected, but activation of these memory cells during a subsequent pregnancy with an RhD-positive fetus results in the formation of IgG anti-RhD antibodies, which cross the placenta and damage fetal red blood cells. The consequences of this process to the fetus include mild to severe anemia, impaired platelet function and dysfunction of the liver and spleen, which can be fatal. The incidence of hemolytic disease of the newborn has fallen dramatically following the introduction of RhD prophylaxis, where anti-RhD antibodies are administered to RhD-negative mothers immediately following the delivery of RhD-positive babies. These antibodies bind to any fetal erythrocytes that may have entered the mother's circulation and destroy them before they can cause sensitization.

Cell-mediated immunity in pregnancy

In contrast to antibody-mediated immunity, maternal cell-mediated immune responses to paternal antigens during normal pregnancy are depressed. As discussed above, this may be as a result of changes in the Th1/Th2 balance in pregnancy. If a shift to Th2-type immune responses is important for the success of normal pregnancy, then it might be expected that this shift does not occur to the same extent in recurrent miscarriage and preeclampsia. This is indeed the case. There is a strong maternal systemic inflammatory response in preeclampsia, as defined by a range of markers (e.g. IFN-γ, C-reactive protein). This exaggerated inflammatory response leads to endothelial

dysfunction in the mother, which is the cause of the maternal syndrome (hypertension, proteinuria, oedema, disseminated intravascular coagulopathy, etc.). This inflammatory response appears to be centred on NK cells rather than T cells [17].

While being a useful working model, the Th1/Th2 model is too simplistic to fully explain the immune changes in pregnancy [17, 18]. It has been found that there is also activation of the mother's innate immune system (monocytes, granulocytes and NK cells) during normal pregnancy, with increased production of Th1 cytokines, IL-12, IL-18 and TNF-α. However, IFN-γ production is not stimulated and there is reduced T cell activity. This must be the pivotal point. It is thought that this inflammatory response is triggered by the release of factors, possibly syncytiotrophoblast microvesicles, from the surface of the placenta into the maternal circulation [17].

Immunotherapy for pregnancy immune disorders

The possibility that immune mechanisms may be involved in implantation failure, recurrent miscarriage and preeclampsia has led some clinicians to use 'immunotherapy' as a possible way of preventing these disorders. There are four main approaches that have been explored.

Paternal and third party leucocyte immunization

The rationale for this controversial treatment is that in women with implantation failure or recurrent miscarriage, exposure of the maternal immune system to paternal antigens during implantation and early pregnancy fails to trigger the necessary facilitating immune response. By analogy with the finding that pre-transplant blood transfusions significantly increase renal allograft survival, its proponents have argued that injecting a preparation of paternal or third party leucocytes into the mother, on one or more occasions prior to conception, will stimulate the appropriate immune response and lead to successful pregnancy. However, there is little scientific basis for this treatment, and meta-analysis of the published studies has shown little benefit.

Intravenous immunoglobulin therapy IVIg

IVIg is produced from pooled human plasma obtained from multiple donors and is used as an immunomodulator in many diseases including autoimmune conditions, transplantation and cancer. Its mode of action is thought to be through antibodies it contains which downregulate B cell and NK cell function. However, there are conflicting data regarding its ability to prevent miscarriage and a number of side effects have been reported. This area requires further study.

Anti-TNFα antibody therapy

Anti-TNFα antibodies are used to treat inflammatory conditions such as rheumatoid arthritis and ankylosing spondylitis. It has therefore been proposed that if implantation failure and miscarriage are due to an excessive Th1 response, this might be modulated by such antibodies. While good success rates for this

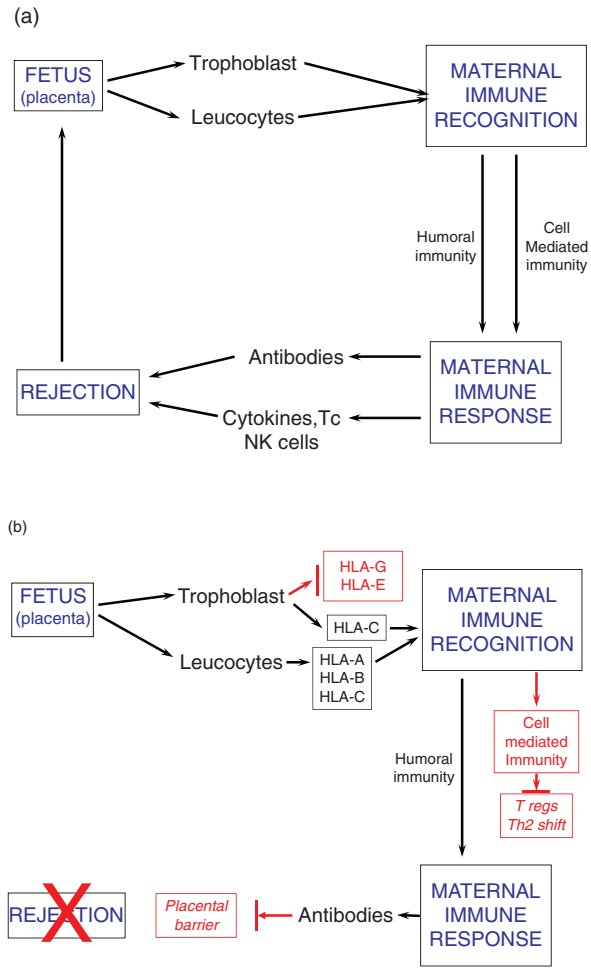

Figure 9.3 Immunoregulatory mechanisms in pregnancy. (a) Immune circuit in pregnancy which could lead to rejection of the placenta and fetus. (b) The immunoregulatory mechanisms which prevent immune rejection of the placenta and fetus. Tc = cytotoxic T cell, NK = natural killer cell

treatment are claimed by some clinics, there is no strong scientific evidence. Furthermore, anti-TNF agents have been reported to be associated with the development of granulomatous disease, lymphoma, SLE and congestive cardiac failure.

Prednisolone therapy

Prednisolone is an immunosuppressive corticosteroid used in the treatment of a wide range of inflammatory and autoimmune conditions. There is evidence that women with recurrent miscarriage have higher levels of uNK cells and increased vascular maturation. This exposes the placenta prematurely to high levels of oxygen which is known to be damaging in early development. Recent studies have shown that administration of oral prednisolone during preconception cycles to women with recurrent miscarriage reduces mid-luteal phase uNK cell numbers and blood vessel maturation [19] and may potentially improve live birth rates, but as is the case for all immunotherapies, it requires further investigation.

Summary

If the maternal immune system was not regulated during pregnancy, then fetal trophoblast and leucocytes would stimulate maternal immune recognition of the placenta and fetus (Fig. 9.3a). This would lead to the production of antibodies and cell-mediated responses which could potentially reject the placenta and fetus. That this does not happen in a normal pregnancy demonstrates that immunoregulatory mechanisms are in place. At the first level, trophoblast HLA-G and HLA-E expression downregulate T and NK cell responses (Fig. 9.3b). However, trophoblast HLA-C and HLA-A,B,C on fetal leucocytes can stimulate cell-mediated and antibody-mediated immunity. Cell-mediated immune responses may be controlled by the Th2 shift and the action of regulatory T cells. However, anti-fetal (paternal) antibodies are produced by the mother, but these are filtered out by the placental barrier and fail to reach the fetal circulation. Thus, by a combination of mechanisms, fetal rejection is avoided.

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Chapter 10

Sperm biology and maturation

William V. Holt and Jane M. Morrell

Sperm maturation in the epididymis

On their release from the testis, spermatozoa pass via the efferent ductules into the head of the epididymis, often described as the *caput epididymidis*. At this stage they are unable to fertilize oocytes, even if removed from the epididymis and placed together with oocytes in a dish. The spermatozoa must, in fact, travel through the whole length of the epididymal duct in order to gain full fertilization competence. Although the epididymal duct is long and complex, for simplicity it is usually regarded as being divided into three regions, which are known either by their anatomical positioning, 'head, body and tail of the epididymis', or by a notation more related to function; i.e. initial, middle and terminal segments. The latter notation is useful when comparing different species where regions of similar function are not necessarily in the same place along the duct. The epididymis and its subdivisions are illustrated in Figure 10.1.

The lack of fertilizing ability upon leaving the testis can be attributed to poorly developed sperm motility, inappropriate chemical composition of the sperm plasma membrane and in some species, such as the guinea pig, even insufficiently differentiated sperm head shape. These immature spermatozoa acquire full fertilizing ability after a period of 10 days–2 weeks, while they make their way along the highly convoluted and coiled epididymal tubule. During their journey along the epididymis they pass through a constantly changing environment whose composition is mainly dictated by the function of the local epithelial cells. One major function of the epithelial cells in the efferent ductules and the head of the epididymis is to extract water from epididymal fluid; passage through these regions causes resorption of about 90% of the fluid [1, 2], and consequently the sperm concentration within the next region (the body of the epididymis, or *corpus epididymidis*) increases significantly. The corpus epididymidis, roughly equivalent to the middle segment, is the region in which the most significant maturational changes are apparent. One obvious morphological modification is the translocation of a small droplet of cytoplasm from an initial position around the neck of the spermatozoon to the distal region of the middle piece. This very obvious change has been used widely as a visual marker of sperm maturation in a number of mammalian species, especially bull, pig and ram, as it was realized many years ago that failure of cytoplasmic droplet migration is correlated with poor fertility. However, Cooper [3] has argued that there should be a distinction between cytoplasmic droplets in domestic species such as the boar and ram, and the abnormal retention of 'excess residual cytoplasm' in human spermatozoa. Although this is also often associated with poor fertility, it does not imply a failure of epididymal function.

As spermatozoa pass through the epididymis they undergo many biochemical changes, especially to the plasma membrane. There is an overall increase in the relative amounts of unsaturated:saturated fatty acids and a decline in the total cholesterol concentration [4, 5]. This has an impact on the biophysical properties of the plasma membrane, making it more 'fluid' and more suitable for participation in membrane fusion interactions such as the acrosome reaction and fertilization itself. The glycosidic composition of the sperm plasma membrane also changes a great deal during epididymal transit, probably as a consequence of the action of glycosides and glycosyl transferases present in epididymal fluid [6–8]. However, recent proteomic studies have established that the protein content of epididymal fluid varies dynamically with respect to

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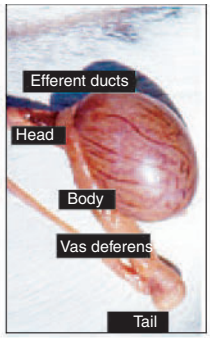

Efferent ducts and Head
Extraction of testicular fluid; concentration of sperm in the tubules
In some species: androgen receptors create locally high androgen concentration

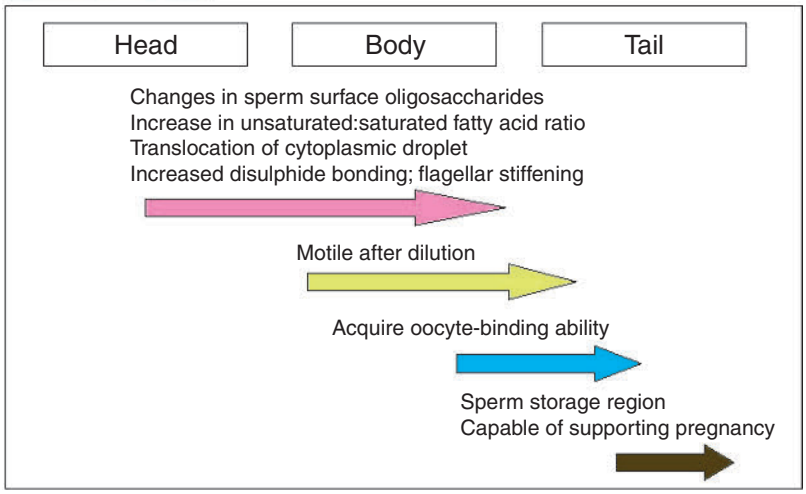

Figure 10.1 The position of the epididymis with respect to the testis, and the major epididymal subdivisions in a typical eutherian mammal (Springbok) are shown, together with a summary of some significant processes that take place within the various epididymal regions.

anatomical position in the epididymal duct, and therefore there is a possibility that post-translational protein insertion into the plasma membrane also occurs [9]. Understanding the nature of the added proteins has been an important objective for researchers interested in developing new forms of contraception, as there is some likelihood that some of the proteins added to the sperm surface in the epididymis could be targets for agents that block sperm-egg interactions.

Various mechanisms for the transfer of proteins to the sperm surface have been identified. As the spermatozoa do not possess active machinery for protein synthesis, they acquire, translocate and modify proteins in relatively unorthodox ways. Epididymosomes are membrane-bound vesicles produced by epithelial cells that transfer proteins directly to the sperm plasma membrane, and it has been shown that the proteins are actually inserted into the membrane rather than simply being adsorbed to the surface [9, 10]. The epithelial cells also produced 'blebs' of cytoplasm that balloon out of the cells. Although these were considered for many years to be artefacts of fixation, more recent studies have shown that they really represent another means of transferring proteins.

Other correlates of sperm maturation include the acquisition of the 'potential' for motility. Although spermatozoa do not show motility while in the epididymal duct itself, they nevertheless acquire the ability to become motile when provided with the appropriate environment. Proteins of the sperm tail become progressively cross-linked by disulphide bonds, with the result that the sperm tails become stiffened and are enabled to exhibit tail bending and hence, motility [11].

Zona binding is clearly an essential first step in fertilization, and since this ability is gained within the epididymis it partially explains how maturation translates into a real acquisition of fertilizing ability. However, zona binding alone is not sufficient to support fertilization; it has to be followed by penetration of the vitelline membrane and formation of the pronuclei. Once that process has been completed, the fertilized eggs actually have to develop normally as embryos. In vitro fertilization experiments with spermatozoa recovered from successively more distal regions of the epididymis have shown clearly that

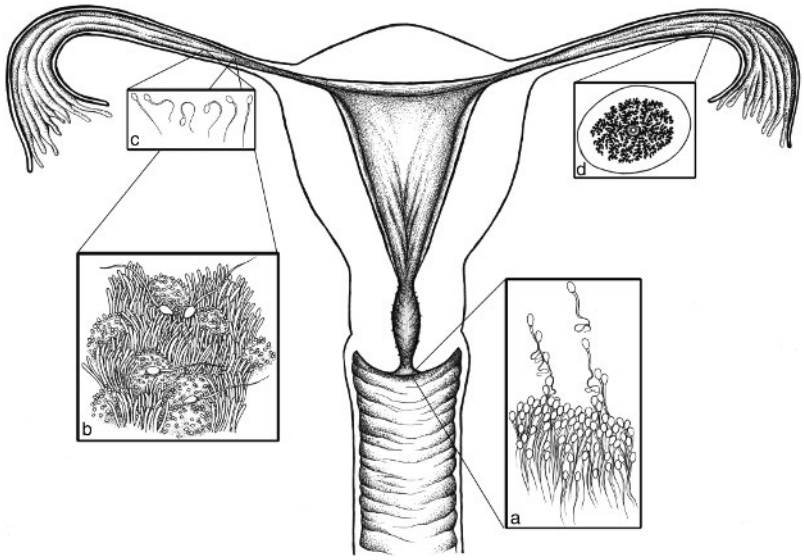

Figure 10.2 Human female reproductive tract illustrating stages of gamete transport. (a) Sperm entering cervical mucus at external os of cervix. The mucus fills the upper half of the inset. (b) Sperm interacting with endosalpingeal epithelium in the fallopian tube. (c) Hyperactivated motility of sperm in fallopian tube. (d) Oocyte in cumulus within a transverse section of the tubal ampulla. The sites of sperm reservoirs and filtration systems are the cervix, uterus and uterotubal junction. Figure drawn by C. Rose Gottlieb, reprinted from Suarez and Pacey, Sperm transport in the female reproductive tract, *Hum Reprod Update* 12 (2006): 25 [1], by kind permission of the authors and Oxford University Press.

this complete level of functionality is not attained until the spermatozoa have reached the tail, or terminal segment, of the epididymis [12].

The tail of the epididymis is also an important site for sperm storage prior to ejaculation in most mammals. In some species, such as sheep, whose normal social mating system involves mating multiple times in rapid succession, the storage region can contain enough spermatozoa for up to 40 ejaculates. In other species, whose social mating systems do not involve the need for large numbers of matings, the epididymal sperm reserves tend to be much lower. Such observations have led to suggestions in the literature that the need for sperm storage capacity has been a prime mover in the evolution of descended testes, as positioning the testes and epididymides outside the body cavity allows the spermatozoa to be stored a few degrees below body temperature [13] where their plasma membranes would be slightly more stable.

Ejaculation marks a transformation in the environment of the spermatozoa and also starts the clock ticking for their eventual demise. From this moment on as they pass along the female reproductive tract they will undergo a series of changes, some of which are irreversible, to equip them for fertilization.

At ejaculation, billions of spermatozoa are released into the female reproductive tract and start to make their way to the site of fertilization – the ampulla of the oviduct. However, only a few thousand spermatozoa are present in the oviducts at any one time, and only one spermatozoon will fertilize each oocyte. Although spermatozoa are present in the ampulla within minutes of semen deposition, these spermatozoa are not the one(s) that will eventually achieve fertilization [14]. They need to undergo a series of maturational changes first that allow them to fertilize the oocyte. Recent research has shown that spermatozoa, far from being inert delivery systems, interact with the female reproductive tract and are subjected to several physiological selection mechanisms responsible for filtering out damaged spermatozoa [15]. This journey in the female reproductive tract is the subject of the current section and is depicted in Figure 10.2.

Seminal plasma

Although the spermatozoa have been kept immotile and quiescent during storage in the epididymis, they are activated by exposure to the secretions of various accessory glands, which together comprise seminal plasma. The purpose of seminal plasma is threefold: to convey the spermatozoa from the male to the female, to allow the spermatozoa to become motile and finally to stimulate the female reproductive tract, both to transport the spermatozoa and to initiate an immune response to deal with bacteria and clear immotile spermatozoa. The components of seminal plasma vary between species: in most primates (including human beings), but few other species, semen coagulates immediately after ejaculation. This coagulate subsequently liquefies over a period of about

30 minutes, gradually releasing spermatozoa to begin their journey towards the oviducts.

As epididymal spermatozoa mature, they develop the capability to become motile when activated. However, it is only when spermatozoa are exposed to the changed ionic environment of seminal plasma that they can actually exhibit progressive motility. Both the bicarbonate ion and the ratio of Na^+:K^+ ions are believed to be important in initiating sperm motility; bicarbonate also plays an important role in capacitation and the acrosome reaction, as described later in the chapter. Extensive studies have shown that the high concentration of bicarbonate in the seminal vesicle fluid compared to epididymal fluid is responsible for stimulating quiescent spermatozoa through direct activation of adenylyl cyclase [16].

Sperm reservoirs and filtration systems within the female reproductive tract

(i) The cervix: Semen deposition takes place in the vagina, close to the posterior cervix, in human beings and some other species, e.g. ruminants and rodents. In other species, e.g. horses and dogs, semen deposition takes place directly into the uterus. In species with vaginal deposition of semen, the cervix acts as a filtration system allowing spermatozoa to escape from seminal plasma, and also serves as a sperm reservoir where the spermatozoa reside for up to 24h. Immotile or morphologically abnormal spermatozoa and bacteria become trapped by the viscous mucus in the cervical lumen and are propelled in a retrograde direction for subsequent expulsion. Normal motile spermatozoa are thought to enter the grooves of the cervical os, making their way along towards the uterine cavity. During the stages of the cycle under progesterone dominance, cervical mucus is much less watery than under estrogen dominance and is nearly impenetrable to spermatozoa.

(ii) The uterus: Although sperm-uterine interaction has been studied mostly in the pig, it is believed that many of the observations in this species are pertinent to other species too. Like the cervix, the uterus seems to act as a sperm reservoir and filtration mechanism, although the latter is based on direct interactions between the spermatozoa and the uterine epithelial cells (UEC). Spermatozoa attached to the UEC show normal ultrastructure and have an existing mitochondrial membrane potential [17], whereas spermatozoa free in the uterine lumen tend to have damaged plasma membranes [18]. These free spermatozoa are exposed to attack by uterine leucocytes and are eventually expelled.

(iii) The utero-tubal junction (UTJ) and distal oviductal isthmus constitute the third sperm reservoir and filtering system. Again, only viable spermatozoa attach themselves to the epithelial cells of these regions and, in human beings, show improved motion parameters [19].

Spermatozoa that have temporarily bound to UTC or the epithelial cells of the UTJ subsequently become free and pass along the oviducts.

Capacitation and hyperactivation

During their sojourn in the sperm reservoirs, spermatozoa undergo a series of membrane changes, the first of which are collectively known as capacitation. These are structural and metabolic changes that allow the spermatozoa eventually to bind to the zona pellucida (ZP) and undergo the acrosome reaction. Without capacitation, spermatozoa cannot bind to the ZP or fertilize the oocyte. The changes are accompanied by an increase in calcium influx, causing raised intracellular Ca^{2+} and cAMP levels, increased membrane fluidity and the acquisition of a new motility pattern known as hyperactivation [20]. The latter is characterized by a change from progressive motility to localized vigorous motility, where the tail beats energetically but the spermatozoa do not move far in any direction, interspersed with periods of relative immotility [21]. Spermatozoa are thought to alternate between hyperactive and progressive motility patterns, possibly in response to chemotactic stimuli, to enable them to locate the oocyte [22]. Although hyperactivation usually occurs during capacitation, the two events are regulated by different intracellular pathways [23]. Hyperactive motility may facilitate the release of spermatozoa from the oviductal sperm reservoir, their passage through mucus in the oviductal lumen and penetration of the cumulus oophorus [24].

Capacitation is difficult to study in vivo because it occurs within the female reproductive tract. However, from in vitro studies it is believed to be initiated by activation of protein kinase A by bicarbonate and soluble adenylyl cyclase [25], leading to the phosphorylation of serine and threonine residues in sperm

proteins. At the same time, cholesterol migrates from the equatorial and post-equatorial region to the apical region of the sperm head, resulting in the activation of tyrosine kinases [26]. There is increasing evidence that sperm capacitation involves an oxidative process and that reactive oxygen species, particularly the superoxide anion (O_2^-), are produced during capacitation in human [27] and bovine spermatozoa [28, 29].

Although the ability to undergo capacitation is a prerequisite for fertilization [30], it was previously thought that, once capacitated, the spermatozoon has a limited life span [31] and that, capacitated spermatozoa are released from the sperm reservoirs in small numbers over a period of time, to enable a small population of spermatozoa capable of fertilization to be present in the oviducts when the oocyte is ovulated. However, recent research suggests that proteins present in pre- and peri-ovulatory oviductal fluid may inhibit capacitation, while those present post-ovulation may speed up the process [32]. These observations suggest that fine regulation of capacitation is possible, enabling the speed of the changes to be modulated or stimulated in accordance with the precise timing of ovulation. It is even possible to 'decapacitate' spermatozoa in vitro and subsequently re-capacitate them after a period of time [33], but the in vivo relevance of these findings is not known.

Sperm-egg interaction: cumulus penetration, zona binding and the acrosome reaction

Sperm penetration of the cumulus matrix surrounding the oocyte in the cumulus oophorus complex is facilitated by membrane-bound hyaluronidase [34] and by their hyperactivated motility pattern. The interaction of the spermatozoa with the cumulus cells is reversible; subsequent interaction with the thick glycoprotein coat surrounding the mammalian oocyte – the zona pellucid (ZP) – is not [15]. Zona binding involves a wide range of sperm surface components and at least one of the three glycoproteins of which the ZP is composed. Zona binding acts as a trigger for the acrosome reaction (AR).

Unlike capacitation, which takes place over a number of hours, the AR is irreversible, fast and, in the absence of zona-binding, is followed by sperm death. Ideally, therefore, the AR should only occur in proximity of the freshly ovulated oocyte, and is stimulated by components of ovarian follicular fluid and proteins of the ZP. The AR is a complex process involving protein phosphorylation controlled by several kinases [27] and bicarbonate may also be involved [35]. Reactive oxygen species (ROS) may prematurely trigger the AR [27].

During the AR, the plasma membrane above the equatorial segment of the sperm head fuses with the outer acrosomal membrane, releasing the acrosomal contents [30]. This exocytosis is triggered by a sustained increase in cytoplasmic calcium concentration. The enzymes released in the AR, coupled with sperm motility, allow the spermatozoon to cut a path through the ZP. The spermatozoon then enters the perivitelline space between the ZP and the oolemma, and the tip of the sperm makes contact with the oolemma, followed by lateral attachment of the spermatozoa [34]. The site of fusion is at the central region of the spermatozoon near, or at, the equatorial region, and involves an integrin on the oocyte and an integrin ligand on the sperm plasma membrane.

For binding to the oolemma to take place, sperm membranes must possess hyaluronic acid binding sites, which are acquired during epididymal maturation. The development of these binding sites in the epididymis is also associated with the acquisition of motility, or at least the capacity to be motile, mentioned earlier in this chapter.

Oocyte activation by sperm fusion results in the exocytosis of cortical granules at the egg plasma membrane. Hydrolases contained in these vesicles act on the ZP, destroying its sperm-binding sites, thereby blocking attempts by other spermatozoa to fertilize that oocyte. The prevention of polyspermy is also achieved in vivo by severely restricting the number of hyperactivated spermatozoa in the vicinity of the oocytes, such that there is approximately one spermatozoon per oocyte [36, 37].

Recently, transgenic mouse lines have been developed expressing green fluorescent protein (GFP) in their acrosome and red fluorescent protein (RFP) in their mitochondria. Since these spermatozoa can be observed through the uterine and oviductal walls, it is hoped that live imaging of the acrosome reaction will soon be possible [38].

Histones, protamines and epigenetics

The majority of proteins in the sperm nucleus, namely histones and protamines, are concerned with packaging and stabilizing the DNA. Sperm chromosomes are not randomly packaged into the nucleus, but occupy

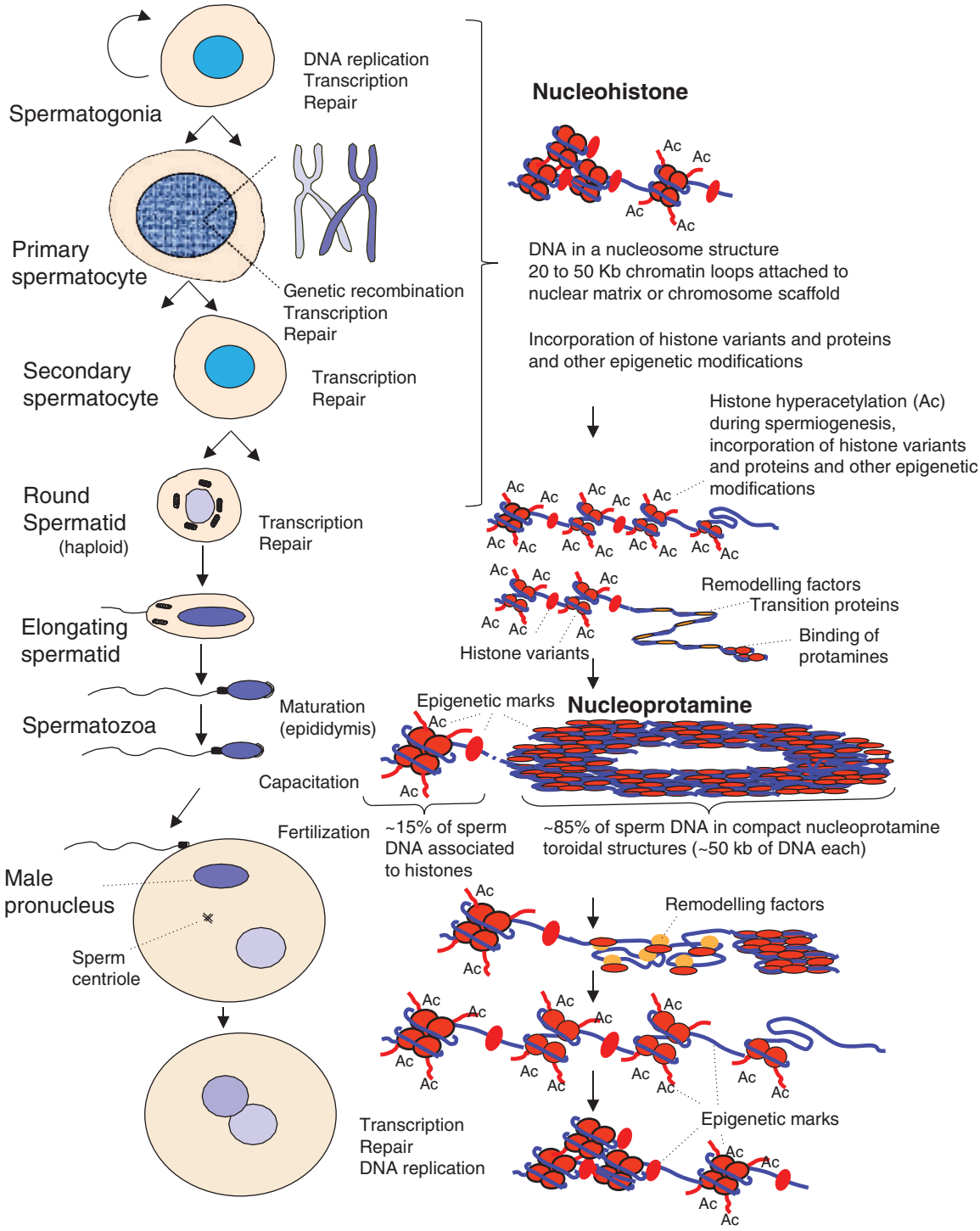

Figure 10.3 Schematic representation of the major cellular, genetic and chromatin changes occurring during spermatogenesis. The left side of the figure represents the cellular changes occurring in the germ cells from spermatogonia (top) to the pronuclear formation after fertilization (bottom). The right side of this figure represents the basic chromatin changes taking place during the nucleohistone to nucleoprotamine transition in spermiogenesis and the subsequent nucleoprotamine unpacking and nucleohistone structure reconstitution at fertilization. The cellular changes in the left side of this figure are intended to correspond roughly to the chromatin structures and activities indicated in the right side. Histones are represented in red colour and DNA is drawn as blue lines. It should be noted that the retention of histones in the mature spermatozoa is very variable in different species. The indicated histone retention in approximately 15% of the sperm DNA corresponds to the situation in humans. Figure reproduced from Oliva *et al., Proteomics* **9** (2009): 1004–17 (Fig. 1) with permission from John Wiley and Sons.

consistent and spatially organized positions, also known as 'territories' [39, 40]. Depending on species, protamines constitute about 85% of the sperm nuclear proteins and have the capacity to package the DNA with extreme efficiency [41]. Histones, which represent the remaining 15% of nuclear proteins, are the same proteins that are found in the somatic cells. While they also interact with DNA and package it in a very specific manner, the histone-DNA complex is relatively loose and gene expression is easier to initiate [42]. The packaging process takes place during spermiogenesis and accompanies the remodelling of the sperm head while the early spermatids develop into spermatozoa. These interactions are illustrated in detail in Figure 10.3 (which was reproduced from Oliva *et al.*, 2009).

DNA itself is known to undergo methylation during spermiogenesis. As this process tends to reduce or 'silence' the transcription of the affected gene sequences, the global methylation process during spermiogenesis is associated with the shutdown of gene expression during this period. The reversal of DNA methylation takes place shortly after the fertilizing spermatozoon enters the oocyte and before the pronucleus is formed. Recent studies have shown that the patterns of sperm DNA methylation closely resemble those of pluripotent embryonic stem (ES) cells and that several embryologically important developmental gene sequences, including members of the HOX gene clusters, are largely unmethylated in both cell types. Imprinting errors in the paternal genome are likely to cause problems to the developing embryos, and there is some evidence for a small increase in imprinting disorders among the offspring of people using in vitro fertilization technologies [43, 44]. Related to this is the finding that an aberrant pattern of DNA methylation in the sperm of some men correlates with known pregnancy failures in their partners [45].

The presence of histones in mature spermatozoa has signalled the possibility that certain sets of genes from the spermatozoa themselves might be amenable to subtle control by epigenetic effects. Sets of different DNA-binding histones form complexes with the sperm DNA; as their DNA binding affinities are variably affected by epigenetic modifications such as methylation and acetylation, this produces considerable variation in the ease with which the DNA coding regions can be read and transcribed. Although this whole topic is complex, it is of considerable interest as it begins to offer some plausible mechanisms for the way in which interactions between the male germ line and environmental influences are inherited for several generations. Experimental studies that involved dosing rats with the fungicide vinclozolin have revealed the potential for induced male reproductive dysfunction to be inherited down four generations [46, 47]. Life history analyses that have investigated relationships between the somatic characteristics of sons and daughters with the environment or behaviour of their parents and grandparents have shown some interesting outcomes that may be epigenetically determined. One specific long-term study involving a population living in the Bristol area of England [48] showed that early paternal smoking (under 11 years old) in fathers was associated with greater body mass index (BMI) at 9 years in sons. This kind of transgenerational association (reviewed by [49]) has received little attention in the context of wildlife, climate change and declining populations, although researchers working on environmental pollution and reproduction are familiar with the scenario [50].

Epigenetic properties of spermatozoa are not limited to the interplay between DNA and nuclear proteins. The existence of mRNA in mature spermatozoa has been viewed largely as a remnant nucleic acid population, left over from the events of spermatogenesis and having no functional significance. However, recent research into the whole issue has provided convincing evidence that the mRNA is transmitting epigenetic information to the next generation. Moreover, comparisons of mRNA profiles from infertile and normally fertile men have shown that important differences exist [51, 52]. Although human spermatozoa are exceptionally rich in mRNA, the fact that it is also commonly present in spermatozoa from other species means that it provides another widely applied source of epigenetic information, and possibly a means for the operation of sperm selection mechanisms inside the female reproductive tract.

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Chapter 11

Fertilization and egg activation

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Introduction

Sexual reproduction is a survival strategy employed by species for procreation, and is characterized by the transfer of haploid genetic material from each parent to produce diploid offspring, ensuring continued genetic diversity. In mammals, males produce vast numbers of gametes called sperm in the testes (~53–55μm in length), which are deposited in the female reproductive tract in a liquid medium (semen) via the process of ejaculation. Females produce much larger gametes, eggs (~100μm in diameter), which are surrounded by a thin glycoprotein layer, the zona pellucida (ZP), and generated in follicles contained in the ovaries which, following maturation, are released and transported through the fallopian tube to the uterus. Sexual reproduction involves the concerted release of these two components, which subsequently fuse to re-form the diploid chromosome number, resulting in a new, genetically unique individual, a process termed fertilization. Fertilization involves a number of key, sequential steps, these are: (1) the acquisition of sperm motility and chemotaxis; (2) capacitation and the acrosome reaction; (3) sperm-egg fusion; and (4) egg activation and the initiation of embryo development. This chapter will summarize these key events.

Sperm motility and chemotaxis

Sperm generated in the testis are immature and immotile, and need to undergo a variety of modifications in order to obtain fertilization competency. The first step of maturation occurs within the testis, where sperm acquire proteins and cholesterols secreted from the epididymis [1]. It is thought that motility is brought about by changes in ambient conditions of the sperm such as temperature, osmotic stimulation, ionic concentration, or following ejaculation, factors secreted from the female reproductive tract [1]. Following the acquisition of motility, sperm begin to make their way up the female reproductive tract towards the egg, employing a process termed chemotaxis.

Chemotaxis is a phenomenon by which single cells or multicellular organisms direct their movements according to certain chemicals in their environment. How human sperm are attracted towards the egg is still a matter of debate, with multiple mechanisms proposed [2]. In some non-mammalian species, peptides or proteins are known to act as chemoattractants (agents of chemotaxis), such as unsaturated fatty acids [1]. In mammalian species, sperm chemotaxis towards follicular fluid has been observed, and progesterone secreted from cumulus cells is thought to be the main chemoattractant [1–3]. Human sperm chemotaxis may also occur via the action of G-protein coupled odorant receptors (OR), such as hOR17–4 [3], on the sperm. Of the total population of ejaculated sperm, not all will be responsive to chemoattraction, due to possible heterogeneity in ORs and responsiveness to chemical chemotaxis, which may serve as a method of selection [1].

Capacitation and the acrosome reaction

Following acquisition of motility, spermatozoa experience further maturation in the uterus, where they undergo a series of physiological, biochemical and biophysical modifications that bestow fertility through a process known as 'capacitation', thought to be conferred by seminal fluid [1]. This process includes alterations in ion concentration, membrane fluidity, membrane hyperpolarization, intracellular pH, protein phosphorylation and concentrations of reactive

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oxygen species [4, 5]. A soluble adenylyl cyclase is also thought to be an enzyme of fundamental importance, activated in the female tract by bicarbonate and calcium, appearing to initiate capacitation [5, 6]. Capacitation is thought to involve membrane-associated factors which prepare the sperm for egg binding and fertilization, and also contribute towards hyperactivation, a phenomenon now recorded in all mammalian species, involving significant changes in flagellum motility [7, 8].

Following maturation and capacitation, the sperm binds to the ZP and undergoes the acrosome reaction. The mammalian acrosome is a highly specialized organelle overlying the sperm nucleus (Fig. 11.1a), containing a number of hydrolytic enzymes required for fertilization. Although the exact function of the acrosome and its contents still remains largely unclear, various acrosomal molecules are considered to be closely implicated in the acrosome reaction or sperm-ZP interaction, and this is also thought to involve an acute rise in Ca^{2+}, the entry of which is thought to be mediated by transient receptor potential channels (TRPCs) [9, 10]. The acrosomal membrane fuses with the sperm plasma membrane (Fig. 11.1b), exposing the contents of the acrosome which contain numerous enzymes, such as hyaluronidase and acrosin, which digest the ZP and the egg membrane [7, 11]. In mice, the ZP consists of three glycoproteins,

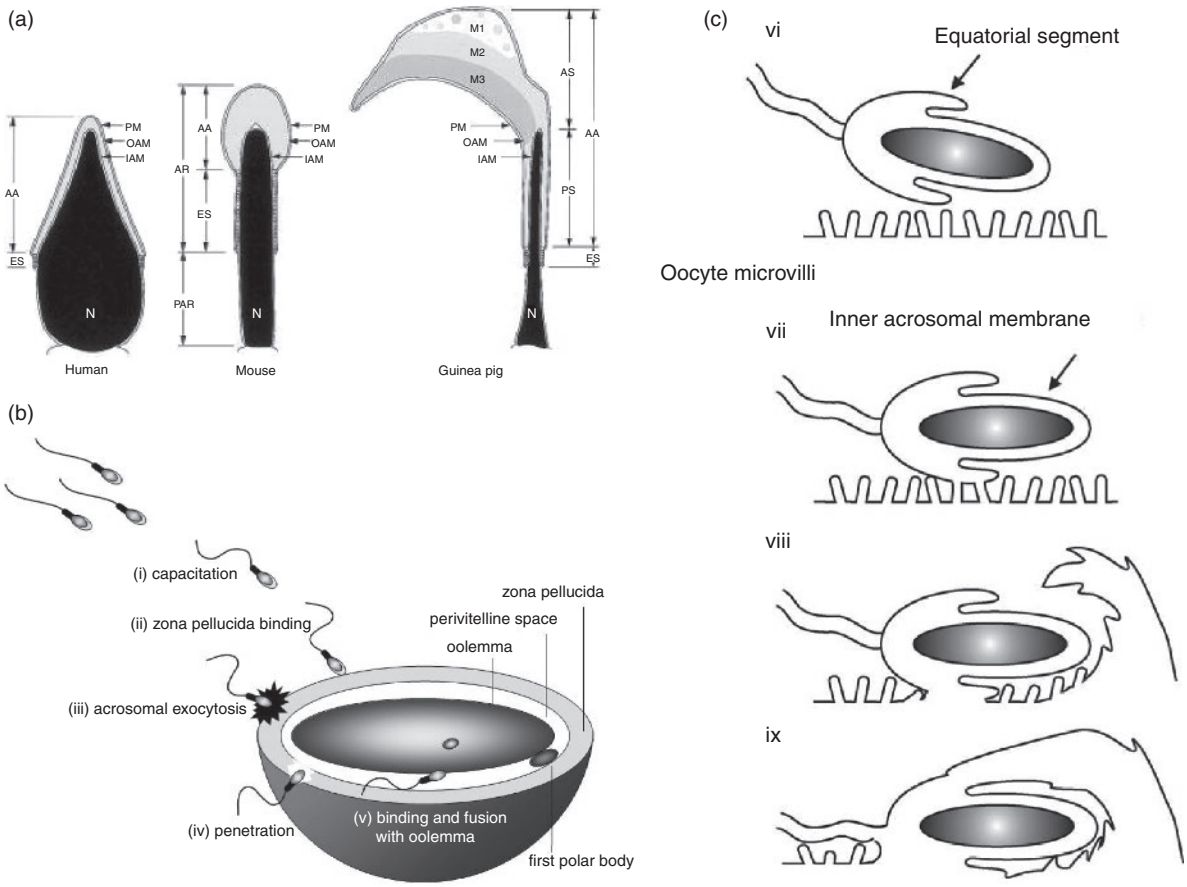

Figure 11.1 (a) Sagittal-sectional views of the heads of mammalian spermatozoa from human, mouse and guinea pig spermatozoa. The acrosomal region (AR) is shaded in grey; it consists of the anterior acrosome (AA) and the equatorial segment (ES). In the guinea pig, the anterior acrosome domain is subdivided into the apical segment (AS) and the principal segment (PS). IAM: inner acrosomal membrane; N: nucleus; OAM: outer acrosomal membrane; PAR: postacrosomal region; PM: plasma membrane. (b) Process of mammalian fertilization. (i) sperm undergo a series of surface and intracellular transformations, collectively termed capacitation, enabling them (ii) to bind to the zona pellucida (ZP) and (iii) undergo the acrosome reaction. (iv) The release of hydrolytic enzymes from the acrosome facilitates sperm passage through the ZP and (v) fusion with the oolemma. (c) Once in the perivitelline space, the fertilizing sperm binds to the microvillar region of the oolemma (vi). In eutherian mammals, fusion with the oolemma begins at the equatorial segment (vii). The posterior region of the sperm head and the tail are subsequently incorporated by the egg via membrane fusion, whereas the anterior region of the head, where the inner membrane is exposed, is engulfed by the egg in a phagocytic manner (viii, ix). Figures adapted from Yoshinaga and Toshimori 2003; Rubinstein *et al.*, 2006; Nixon *et al.*, 2007.

referred to as ZP1, ZP2 and ZP3, with sperm-binding activity residing on serine/threonine-linked oligosaccharide chains of ZP3, thought to contribute towards Ca^{2+} influx in sperm and to bind sperm surface b1,4-galactosyltransferase-I (GalT) [10, 12, 13]. Several lines of evidence also point towards phosphotidylinositol signalling pathways being implicated in the initiation of the acrosome reaction, most particularly phosphotidylinositol-3-kinase (PI3K) [10].

Following capacitation and the acrosome reaction, the sperm proceeds to fuse with the egg plasma membrane in preparation for fertilization [Fig. 11.1c). In general, acrosomal exocytosis is thought to be required for successful fusion of the sperm with the egg. Following acrosomal exocytosis, proteins coating the inner acrosomal membrane are exposed, possibly to mediate interactions between the sperm and the ZP during penetration [14].

Sperm-egg fusion

Membrane fusion requires a mechanism in which two lipid bilayers are transformed from two separate barriers into a single lipid bilayer, in this case involving fusion between the sperm and egg membranes. This is thought to involve a variety of receptor/ligand interactions, on both the sperm and the egg.

Sperm ligands

The ADAM (A Disintegrin And Metalloprotease) family of integral membrane proteins are thought to play a major role in sperm-egg fusion [12]. A large set of ADAMs are expressed in mouse testis, including ADAM-3/cyritestin. Peptides derived from the disintegrin domain of ADAM3/cyritestin were shown to inhibit sperm binding and fusion [15]. Another candidate is thought to be a testis-specific protein, *Izumo*, only detectable on acrosome-reacted sperm. *Izumo* knockout male mice are sterile, despite normal mating behaviour and ejaculation [15]. Of note is the ability of *Izumo* to bind identical or closely related immunoglobulin family members [14]. The testis specificity of *Izumo* makes its interaction with a related cell adhesion molecule (CAM) protein on the oolemma a distinct possibility [14]. Further candidates include protein DE (acidic epididymal glycoprotein), a member of the CRISP (cysteine-rich secretory proteins) family. The human epididymal sperm protein ARP (AEG-related protein) is a member of this family, and is tightly associated with the sperm surface [12, 14, 15].

Egg ligands

There are several key players within the egg that are involved in sperm-egg fusion. First, a glycosyl phosphatidylinositol (GPI)-anchored protein may be required, removal of which results in eggs with severely reduced ability to fuse with sperm [12, 16]. A second candidate is the tetraspanin protein, CD9, knockout of which results in almost complete loss of sperm-egg fusion ability. So far, the mechanism of CD9 function in gamete fusion has not been clarified, but it may act in coordination with other egg surface proteins. Intriguingly, the activity of a Zn21-dependent metalloprotease is also considered to be involved, the identity of which, or which gamete expresses it, is not known [12, 15, 16].

Following the events of gamete fusion, the egg primes itself for a major event of fertilization. Eggs are stocked with maternal RNAs and proteins required to support embryogenesis, but are not transcriptionally active. Instead, the egg nucleus is arrested at the metaphase of the second meiotic division, following the exclusion of the first polar body at egg maturation. All these features must be modified to allow embryogenesis following fertilization. The process that accomplishes this is called 'egg activation' [17].

Egg activation

Egg activation consists of a series of concurrent events. The egg membrane undergoes physical and chemical changes to prevent polyspermy and to protect and support the embryo. In many animals, this occurs following sperm-egg recognition at the ZP, and polyspermy is blocked following biophysical changes in the egg membrane. Other strategies include the cortical reaction, which involves the release of cortical granules, which traverse to the plasma membrane, causing biophysical changes in the ZP [17].

In most mammals, meiotic arrest is released to allow the cell cycle to complete, forming a haploid female pronucleus that is capable of combining with the sperm-derived male pronucleus. Meiotic arrest is established and maintained by stabilization of the universal driver for the G2/M transition, the M-phase promoting factor (MPF). MPF is a heterodimer composed of a regulatory cyclin subunit, cyclin B, and a catalytic subunit, Cdc2 kinase. Active Cdc2 drives entry into M-phase by phosphorylating substrates that lead to nuclear envelope breakdown and spindle formation [17]. The nucleus delivered by the sperm

then undergoes a process of remodelling to permit DNA replication and fusion with the female pronucleus. The sperm nuclear envelope is then removed, and sperm-specific protamines or histones are replaced by histone variants from maternal stores [17].

The populations of maternal mRNAs and proteins undergo several dynamic changes, including regulated degradation, translation or post-translational modifications. The duration and initiation of these events differ between species, beginning at day 1 (2-cell stage) in the mouse. Cytoskeletal rearrangements occur, presumably to support zygotic growth and development. The ultimate aim of these events is the cellular transformation of a mature egg into a developing embryo [17].

The exact mechanisms by which the sperm causes these changes in the mammalian egg were a subject of intense debate, until the discovery that activation also involves repeated oscillations of free cytosolic calcium (Ca^{2+}) [18]. In contrast, oocyte activation in non-mammalian species such as sea urchins and frogs is triggered by a single Ca^{2+} transient [19].

The role of calcium at egg activation

The first evidence for the importance of Ca^{2+} signals in oocyte activation came from the application of Ca^{2+} sensitive fluorescent dyes to eggs from a wide range of species, leading to the association of increased cytosolic Ca^{2+} and the initiation of activation/embryogenesis (Fig. 11.2a). Microinjections of Ca^{2+} alone sufficiently triggered embryo development in mice [18]. Other studies showed that the temporal pattern of Ca^{2+} oscillations was species-specific, with different species possessing specific patterns of amplitude, duration and frequency over time (Fig. 11.2b, c) [20]. It is generally

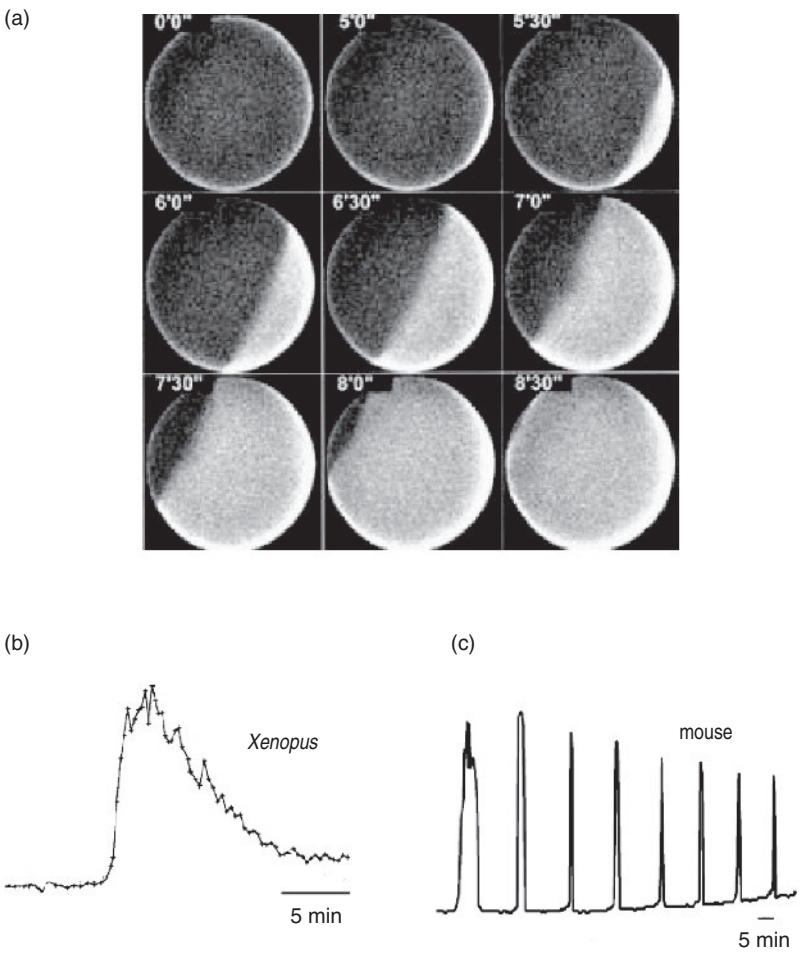

Figure 11.2 (a) Calcium wave as observed using calcium-green-1-dextran in a *Xenopus* egg. Time 0 shows the egg's resting levels of calcium. The sperm-induced calcium wave is initiated by sperm entry (indicated by white arrow), following which the wave traverses the entire egg. (b) The temporal sequence of calcium waves in *Xenopus* eggs and (c) mouse oocytes. Figure adapted from Fontanilla and Nuccitelli 1998, and Miyzaki 2006 with permission.

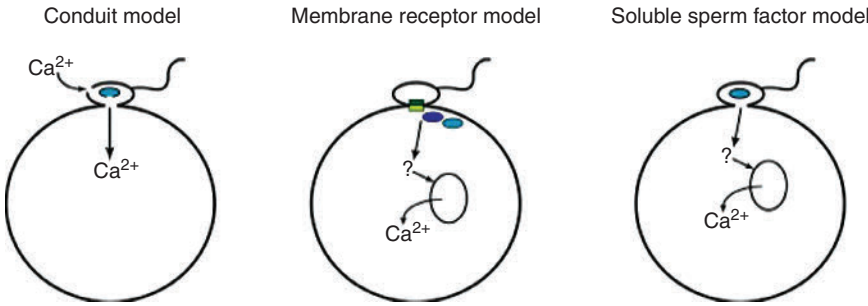

Figure 11.3 The three major models of egg activation. The conduit model suggests that Ca^{2+} enters the egg via a Ca^{2+} channel opening in the sperm plasma membrane; the membrane receptor model suggests that a receptor activated by sperm binding relays a signal to initiate the Ca^{2+} release mechanism, and the soluble sperm factor model suggests that sperm deliver a molecule that initiates the Ca^{2+} release mechanism. Figure adapted from Parrington *et al.*, 2007.

acknowledged that these Ca^{2+} oscillations are a direct result of inositol triphosphate (IP_3) mediated Ca^{2+} release [18, 21]. Cytosolic IP_3 concentrations increase during fertilization in mammalian eggs [18], emphasizing the importance of IP_3 levels and IP_3-mediated Ca^{2+} release. However, the precise mechanism underlying these oscillations has been the subject of intense debate, specifically in relation to the relative roles played by the sperm and egg. Three predominant models have been proposed: (1) the Ca^{2+} conduit model, (2) the membrane receptor model, and (3) the soluble sperm factor model [21] (Fig. 11.3).

Models of egg activation

The Ca^{2+} conduit model

This model stated that the sperm may introduce a bolus of Ca^{2+} directly into the egg following fusion, leading to Ca^{2+} oscillations being initiated at egg activation. This was further modified to state that following sperm-egg fusion, sperm membrane channels may act as conduits, allowing Ca^{2+} entry directly into the egg, thus inducing Ca^{2+}-mediated Ca^{2+} release. While an attractive model, IP_3-mediated Ca^{2+} release is a specific requirement of egg activation. The injection of Ca^{2+} directly into eggs failed to induce further Ca^{2+} increase, and studies of mouse eggs showed no increase in local cytoplasmic concentrations of Ca^{2+}during gamete fusion. Furthermore, sea urchin eggs can be activated by acrosome-reacted sperm in seawater lacking Ca^{2+} [21].

The membrane receptor model

Initially the most popular theory, this model proposed that egg activation may be triggered following interaction between a sperm ligand and a corresponding egg receptor, in turn activating a signalling cascade which leads to the activation of an egg phospholipase C (PLC). This was the dominant hypothesis for many years, adopting fundamental features of somatic cells, where such surface-mediated interactions are the classic mechanism for signal transduction. However, a sperm ligand/egg receptor mechanism of this type has yet to be shown to be the trigger for egg activation, despite numerous candidate molecules being investigated. Furthermore, the successful nature of intracytoplasmic sperm injection (ICSI), whereby sperm is directly injected into the egg thus bypassing any membrane pathway, casts significant doubt over the existence of a sperm ligand/egg receptor mechanism in operation during egg activation [21].

The soluble sperm factor model

The sperm factor model proposed that egg activation may be triggered by a soluble factor released from the sperm into the egg during, or immediately following, gamete fusion [22]. The first evidence for the presence of a soluble sperm factor came from injection of sea urchin sperm extracts, which induced egg activation, followed by similar observations using ascidian sperm [21, 23]. Evidence for the existence of a soluble sperm protein able to mimic the pattern of Ca^{2+} release seen at fertilization emerged from a series of studies in which sperm extracts were injected into eggs harvested from a variety of mammals, including marine worms and ascidians [21, 23]. Sperm extracts from frogs, chickens and tilapia fish have also been shown to trigger Ca^{2+} oscillations in mouse eggs, providing further credence that a soluble sperm factor is responsible for egg activation, not only in mammals, but in a wide range of species [21].

Identification of PLCζ as the sperm factor

The precise identity of the sperm factor is still a matter of intense debate, with various candidates proposed for the endogenous agent of egg activation. One such candidate is the post-acrosomal sheath WW domain-binding protein (PAWP) [24]. It is possible that in some species there is not one particular oocyte activation factor, but a combination of factors acting together [24].

However, at least for mammals, a major breakthrough in our understanding of the identity of the endogenous sperm factor came with the discovery of a soluble sperm-derived phospholipase C, PLCζ, with distinctive properties [25]. Injection of both recombinant mouse PLCζ RNA and protein into mouse oocytes resulted in the initiation of Ca^{2+} oscillations similar to those seen at fertilization, and embryonic development to the blastocyst stage. Immuno-depletion of PLCζ from sperm extracts suppressed Ca^{2+} releasing ability, while sperm fractionation studies indicated that the presence of PLCζ in sperm correlated to the sperm's ability to induce Ca^{2+} oscillations in the oocyte. Furthermore, RNA interference (RNAi) experiments produced transgenic mice with significantly reduced expression of PLCζ in the testis. Fertilization by sperm from these animals was characterized by the premature cessation of Ca^{2+} oscillations within the oocyte. PLCζ appears to play a similar role during fertilization in non-mammalian species such as the chicken, medaka fish and quail, suggesting the existence of a mechanism common to all vertebrates [26].

While many now agree that PLCζ is the trigger of egg activation in mammals [21], the egg itself also appears to play a pivotal role in the activation mechanism. Ca^{2+} oscillatory ability is acquired following successful oocyte maturation and involves various cytoplasmic changes [27]. Ca^{2+} transients in fertilized immature mouse eggs are of lower amplitude than eggs fertilized in metaphase II (MII). An increase in the number and sensitivity of IP_3 receptors results in an increase in Ca^{2+} release [28–30]. Recent findings also suggest that egg mitochondria also play an instrumental role through the endoplasmic reticulum and IP_3 mediated Ca^{2+}-signalling (Fig. 11.4) [31, 32].

PLCζ as a fundamental agent of egg activation

PLCζ exhibits typical PLC domain structure and has closest homology with PLCδ isoforms, particularly with PLCδ1. PLCζ possesses characteristic X and Y catalytic domains which form the active site common to all PLCs, a C2 domain and a set of EF hands. One major difference is the absence of a PH domain in PLCζ, which is also the smallest known mammalian PLC. Alignment of the catalytic domains of PLCζ and PLCδ1 revealed that active site residues are conserved, or conservatively replaced. Mutagenesis of the PLCζ active site leads to the loss of its ability to cause Ca^{2+} release, confirming that as with PLCδ1, the active site of PLCζ is responsible for targeting PIP_2 to cause IP_3-mediated Ca^{2+} release [23].

PLCζ is also distinctive compared to other PLC isoforms in its high sensitivity to Ca^{2+}, explaining why PLCζ is much more effective than other PLCs at causing IP_3 production and Ca^{2+} release in the oocyte cytoplasm. Mammalian PLCζs have four EF-hand motifs at their N-termini, which appear to play an important role in enzyme activity. Without a PH domain, it currently remains unclear how PLCζ targets its membrane-bound substrate phosphatidylinositol 4,5-bisphosphate (PIP_2), since in other PLCs the PH domain serves to anchor the enzyme to specific plasma membrane proteins. It is possible that the C2 domain may aid PLCζ in targeting membrane-bound PIP_2. Deletion of the C2 domain of PLCζ leads to some loss of enzymatic activity and only a slight reduction in Ca^{2+} sensitivity. However, injection of cRNA corresponding to PLCζ lacking the C2 domain into mouse oocytes failed to elevate Ca^{2+} levels, implying that the C2 domain is also required for the ability of PLCζ to initiate oocyte activation [23].

The other non-catalytic domain of PLCζ that may regulate its activity is the segment between the X and Y catalytic domains, the X-Y linker. The proximity of the X-Y linker to the active site indicates some potential involvement in regulating catalytic activity, or PIP_2 binding. It has been suggested that a cluster of unstructured basic residues within the X-Y linker may be responsible for membrane binding, or even attracting PIP_2, sequestering it in the locality of PLCζ, in an electrostatic manner. However, unlike PLCγ, the PLCζ X-Y linker does not contain any regions of predicted secondary structure, raising the question of how it would play a role in anchoring PLCζ to PIP_2. There is also evidence to suggest that PLCζ remains functional following proteolytic cleavage at the X-Y linker, although data suggest that these fragments can re-form complexes to retain activity. Intriguingly, a recent study indicated that the X-Y linker in most, if not all PLC, isoforms plays an auto-inhibitory role, the

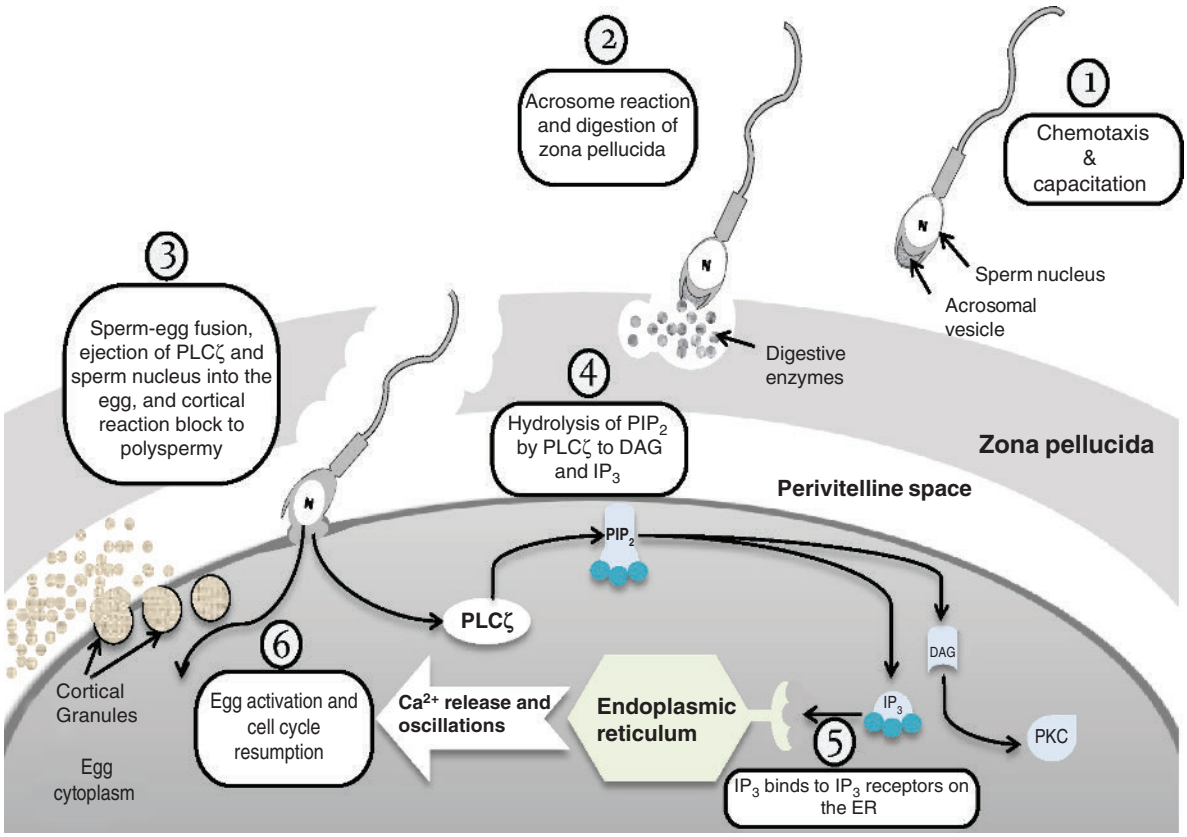

Figure 11.4 Signalling pathway of PLCζ.

deletion of which results in elevated activity (for reviews, see [21], [23], [26]).

Following fertilization, oocyte Ca^{2+} oscillations cease at the time of pronuclei formation with subsequent oscillations then being observed in mouse zygotes during mitosis. One possible explanation for such cell-cycle dependent termination and resumption is that PLCζ localizes to the pronuclei during interphase, resulting in the cessation of Ca^{2+} oscillations, resuming following pronuclear envelope breakdown during the fertilized egg's entry into mitosis. That PLCζ localizes to the pronucleus is suggested by it possessing a nuclear localization signal (NLS) and directly supported by observations that mouse PLCζ tagged with either a Venus fluorescent protein or a Myc epitope can be seen associated with nascent pronuclei. Tagged PLCζ was also observed to return to the cytoplasm during the first mitosis in a manner that coincided with the resumption of Ca^{2+} oscillations. It remains possible that there is a role for PLCζ within the nucleus itself. However, human PLCζ does not translocate to the pronucleus when injected into mouse oocytes, so the relevance of these findings in terms of human fertilization remains to be proven [26].

PLCζ appears to vary in amount and degree of solubility among mammalian species [23]. Different species may deliver 'fine-tuned' doses of PLCζ, which may have varying potencies adjusted via evolutionary processes to match the size and sensitivity of the egg being activated [23, 33]. Studies involving the localization pattern of PLCζ in sperm have provided valuable information with regards to investigation of PLCζ function. PLCζ in non-capacitated human sperm was predominantly localized to the equatorial region of the sperm head, and this pattern was maintained during capacitation and the acrosome reaction [34]. This location would be an ideal one for a sperm factor, allowing rapid release into the egg following gamete fusion. However, as well as this predominantly equatorial pattern of localization, PLCζ was also detected to a lesser extent in acrosomal and post-acrosomal regions of human sperm. Recent studies

suggest that PLCζ RNA transcripts are present within human sperm, and it has been suggested that such transcripts could be transcribed during fertilization. However, the functional significance of these PLCζ transcripts, as for all RNA transcripts present in human sperm, remains to be verified [26].

Intriguingly, however, the pattern of PLCζ immunofluorescence within the post-acrosomal region in human sperm changed during capacitation [34], suggesting that changes within PLCζ structure or its interactions with other cellular components could occur. A second population of PLCζ has also been detected in the acrosomal region, suggesting that PLCζ may be involved in other roles besides egg activation, such as the acrosome reaction [35, 36]. Various PLCζ isoforms have been detected within porcine sperm, ranging from the expected full length 74 kDa, to isoforms ranging in size from 27 kDa to 50 kDa, which may exist in stable complexes, retaining enzymatic activity [37]. Similar patterns are also detected in human sperm [38], but the functional significance of these alternative forms has yet to be ascertained.

Fertilization failure and infertility

Fertilization is an essential process in the procreation of sexually reproducing species such as humans. Given the complex nature of the processes involved, defects in any one, or a combination of defects, in the aforementioned steps could result in fertilization failure. Failed fertilization due to sperm-related factors has been related to deformed sperm nuclear morphology, acrosomal defects, centrosomal dysfunction and chromatin status [39–42]. An example of a known condition where multiple variables within the sperm are affected is globozoospermia (round-headed sperm), a condition affecting 0.1% of infertile men in which acrosome formation and oocyte activation capacity are abnormal [43]. Sperm from globozoospermic men routinely fail ART procedures such as IVF and ICSI [44]. Sperm-egg fusion is also vulnerable to disruption, and defects in sperm or egg membrane receptors such as ADAMs or *Izumo* can lead to infertility in knockout mice, due to the inability of the sperm to interact succesfully with the egg [3], although the relevance of these in humans remains to be ascertained. Recent data also suggest the involvement of sperm DNA fragmentation in fertility. DNA abnormalities are not repaired by the egg following fertilization, and are likely to result in impairment of embryo and fetal development [45]. Defects in factors which regulate early embryo development following the main events of fertilization may also contribute towards fertilization failure, such as MATER (Maternal Antigen That Embryos Require), or the failure of the embryonic genome to activate [46].

Current ART methods are able to rectify most forms of infertility. For example, abnormal ovulation may be treated by in vitro maturation (IVM). Similarly, ICSI can rectify a number of male factor problems, such as oligospermia (low sperm count). Although ART plays a critical role in the reduction of infertility, ART has not yet proven successful for some groups of infertile couples. Of particular outstanding concern are infertile males exhibiting unexplained (idiopathic) male factor infertility, or egg activation deficiency (for review, see [26]).

Clinical relevance of fertilization failure

Infertility represents a growing problem to an increasing proportion of the population, leading governments worldwide to invest heavily in ART, which not only has led to significant improvements in our understanding of male/female reproductive systems, gamete preservation and gamete manipulation, but also represents 7% of all births in some developed countries, with more than approximately 5 million ART babies born worldwide. It is estimated that male factor infertility contributes to 35–40% of infertility in couples, depending upon the biogeographical region studied, the leading cause of which is widely accepted to be due to abnormalities in testicular gene expression [26].

Markers of sperm function are increasingly being proposed for study, to more efficiently replace current clinical procedures and to generate novel diagnostics and therapies to combat forms of idiopathic male factor infertility. Such examples include specific pathways in capacitation, such as soluble adenylate cyclase (sAC), and sperm-egg fusion markers such as ZP3 [39]. Diagnostics such as genetic screening for testicular genes involved in sperm function have also been proposed, such as the azoospermia factor (AZF) gene. Conversely, such markers are also being utilized to produce therapeutics for a reversible male contraceptive, which are not as invasive as current surgical techniques, or unreliable as hormonal contraception due to side effects or functional delay, and specifically target sperm function. Examples include specific inhibitors for sAC, or sperm channels such as SLO3

or CATSPER [47]. However, relatively little is known about such markers, and a significant amount of research is still required in order to achieve a viable diagnostic and/or therapeutic. Furthermore, therapeutics against these targets modulating fertility would exclude forms of male factor infertility where egg activation is deficient. It is with this regard that much promise is shown by PLCζ.

Links between PLCζ defects and egg activation failure

Although ICSI results in average successful fertilization rates of 70%, complete or virtually complete fertilization failure still occurs in 1–5% of cases. The incorrect injection of sperm, expulsion of injected spermatozoon from the oocyte and the failure of sperm head decondensation are not considered to contribute substantially to fertilization failure after ICSI. Instead, a deficiency in the mechanism of oocyte activation is regarded as the principal cause of fertilization failure, or abnormally low fertilization after ICSI (for review, see [26]).

Given the proposed role of PLCζ in oocyte activation, it is possible that abnormal forms or aberrant function of PLCζ may be the underlying cause of certain types of male factor infertility where egg activation is deficient. Sperm of infertile men that consistently fail IVF and ICSI are unable to produce Ca^{2+} oscillations upon injection into mouse oocytes, or those that do are uncharacteristic of oscillations observed from fertile men, reduced in both frequency and amplitude [38, 48]. Furthermore, immunofluorescence and immunoblot analysis have revealed that infertile patients whose sperm failed ICSI and were unable to produce Ca^{2+} oscillations, showed abnormalities in PLCζ expression (Fig. 11.5) [38, 48, 49]. Moreover, the activating ability of egg activation-deficient human sperm following ICSI was rescued upon co-injection with mouse PLCζ mRNA [48].

Although analysis of the PLCζ gene revealed no major genetic abnormalities in globozoospermic (round-headed, acrosome-less sperm) men, the sperm of these patients exhibited reduced levels or absence of PLCζ expression [38, 48, 49]. Interestingly, following the use of ICSI along with a Ca^{2+} ionophore, high rates of fertilization were observed along with an ongoing pregnancy in a multitude of cases where egg activation was deficient (for review, see [26]). Importantly, Heytens *et al.* (2009) identified a heterozygous substitution mutation in the coding sequence of PLCζ in a non-globozoospermic infertile male, which was thought to disrupt protein structure and function, as confirmed

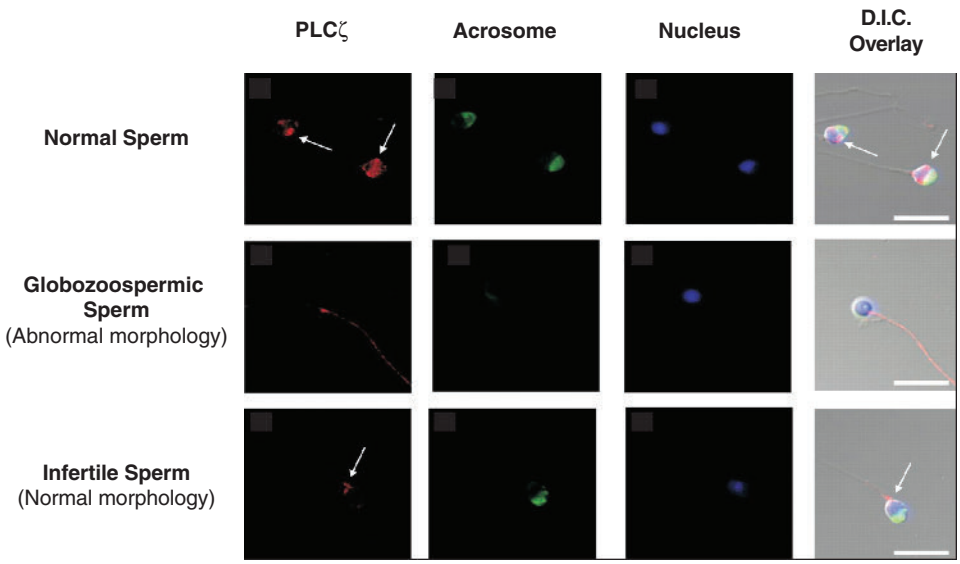

Figure 11.5 Immunocytochemistry of PLCζ (red) in a sperm from a fertile man (top panel) illustrating immunostaining in the equatorial region (arrows), in a globozoospermic man (middle panel) showing a reduction/absence of PLCζ in the sperm head, and in morphologically normal but infertile sperm (bottom panel), with reduced/absent PLCζ levels. FITC-PSA-lectin staining (green) identifies the acrosome, while sperm nuclei were identified with Hoechst 33 342 (blue). Scale bars indicate 5 μm. Figure adapted from Heytens *et al.*, 2009 with permission.

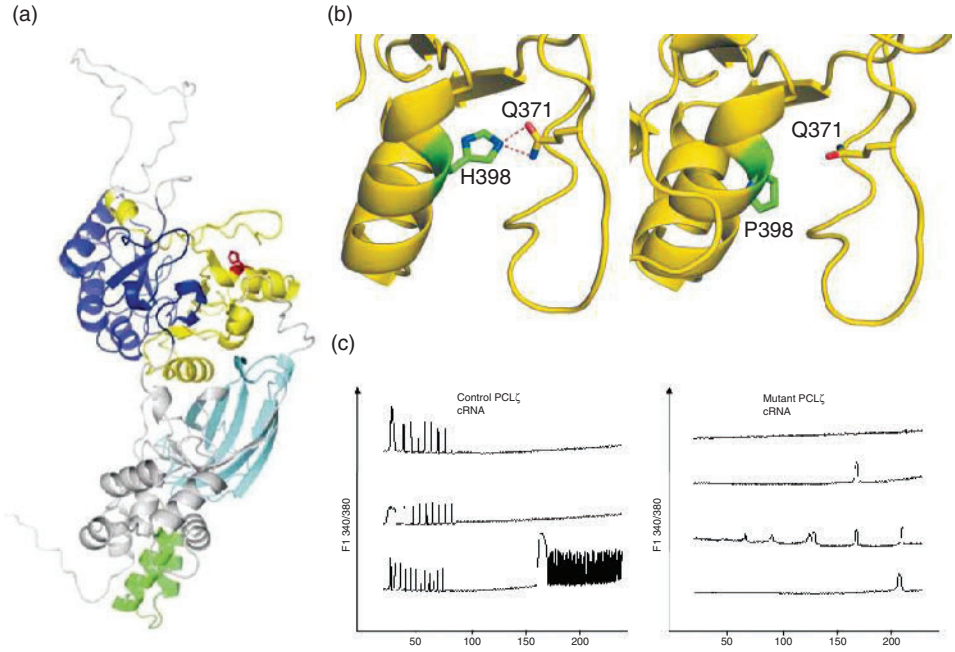

Figure 11.6 Histidine>Proline point mutation identified in an infertile male patient diagnosed with egg activation deficiency. (a) Model of human PLCζ showing functional domains (green – EF hand, blue – X catalytic domain, yellow – Y catalytic domain and cyan – C2 domain). Histidine398 is shown in red. (b) Close-up of H398 showing side-chain-side-chain hydrogen bonds alongside a close-up of P398 in mutant PLCζ showing no side-chain-side-chain hydrogen bonds. (c) Microinjection of wild type and mutant PLCζ into mouse oocytes and resulting calcium release patterns.

by modelling and cRNA injections into mouse eggs (Fig. 11.6a–c) [38].

As PLCζ is now widely considered to be the physiological agent responsible for egg activation in mammals, it represents both a novel diagnostic biomarker of egg activation and a possible mode of treatment for certain types of male infertility. PLCζ localization within the sperm may aid in the identification of some forms of male factor infertility. While egg activation failure can be overcome by artificial egg activators such as calcium ionophores, purified recombinant PLCζ protein has potential as a more physiological agent of egg activation. It may also be beneficial to develop cell-free and enzymatic tests for PLCζ activity to diagnose infertility linked to egg activation deficiency. The identification of men with egg activation defects also raises the possibility of using recombinant PLCζ protein as a potential therapeutic with which to modulate forms of infertility where egg activation is an issue [26]. Therefore, while much work is still needed with regards to factors such as PLCζ, ZP1–3, or CATSPER, as our understanding increases with regards to the intricate network of events that occur during fertilization, egg activation and early embryo development, so do the chances of utilizing such markers clinically and diagnostically.

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Chapter 12

Early embryogenesis

Shankar Srinivas and Tomoko Watanabe

Overview and general concepts

The process of embryogenesis is the basis for establishing the final form of the adult. Embryonic development does not consist simply of the growth in size of a 'preformed' miniature fetus, but is a tremendously dynamic process, characterized by a great deal of cell movement and tissue rearrangement. An embryo starts out as a single cell, the fertilized egg, which divides to form a mass of cells. Over the course of embryogenesis, a pattern emerges from this initially inchoate collection of cells. This pattern is manifest as changes in cells, resulting in the generation of different types of tissues, and in the stereotypic arrangement of these cells and tissues to generate recognizable fetal form.

Coordinated with the morphogenetic events that shape the embryo is the progressive *differentiation* of cells, to achieve functional specialization. Differentiation is generally accompanied by a reduction in the *potential* of those cells (in terms of cell types they can subsequently differentiate into). Embryogenesis starts with the zygote, said to be *totipotent* because it can give rise to all fetal and placental tissues. The zygote undergoes repeated rounds of division to yield cells that differentiate into *pluripotent* cells, which can give rise to many cell types (including all those found in the fetus), but not certain cell types that make up the placenta. In the course of embryogenesis, these pluripotent cells differentiate further, giving *multi, oligo* and *unipotent* cell types. Some of these cell types can be described as *stem cells*, capable of indefinite self-renewal (given the appropriate *niche*), while also retaining the potential to differentiate into specific cell types.

Differentiation is mediated by changes to the gene expression profile of cells. These changes are brought about by many different mechanisms, such as the differential inheritance of asymmetrically localized cytoplasmic determinants during cell division or in response to *inductive* signals from neighbouring cells. Such mechanisms set off a cascade of events that ultimately lead to specific genes being activated or repressed, defining the gene expression profile of the cell. Networks of genes that guide development are frequently reused in different contexts. It is therefore not uncommon for a specific gene to play an important role in the development of completely different structures – for example, the gene *Sonic Hedgehog* (*SHH*) is key to the proper patterning of the neural tube as well as the limb.

This chapter gives a broad overview of the major developmental events that take place during early embryogenesis, from fertilization up to the formation of the first organs. Preimplantation development is considered first, during which the embryo undergoes the first differentiation events essential in delineating placenta and the fetal lineages. The next major event is gastrulation, during which the fundamental tissues types used to build the embryo are generated. The chapter ends with a description of embryonic turning, the process by which the initially flat embryo is converted to a tubular form. These areas are per force described in brief, but the reader is referred to several excellent texts for greater detail on specific topics.

Preimplantation development

Placental mammals are unique in developing inside a womb, which first requires the formation of a placenta as an interface between the mother and fetus. The early days of embryogenesis therefore are devoted to the formation of cell types that can be used to contribute to the placenta. An oocyte is full of maternally encoded mRNA and proteins, stored during oogenesis. This stored material enables the zygote to jump-start embryonic development as soon as fertilization occurs. The loss or depletion of maternal factors causes embryos to

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stop development. For example, the lack of heat shock factor (HSF1) results in one-cell arrest.

The embryo depends on maternal transcripts until around the two-cell stage, when its genome is activated in a process called *maternal-to-zygotic transition* (MZT). This occurs in two phases – first, a subset of maternal transcripts is eliminated, then in the second phase, zygotic transcription is initiated. During MZT, both maternal and zygotic transcripts are active. The majority of maternal transcripts are degraded by the end of the two-cell stage, when the switch to zygotic transcripts is finished.

After fertilization, the zygote undergoes a series of *cleavage divisions* – cell division without growth – resulting in a progressive reduction in cell volume. As cleavage proceeds, the individual cells are now called blastomeres, and form a *morula* (from the Latin for mulberry, because of the distinctive appearance of the clump of cells).

Cleavage division is relatively slow at first, so that on day 1 after fertilization the embryo consists of two blastomeres, on day 2 of four blastomeres and on day 3, the embryo consists of eight blastomeres (Fig. 12.1). Separation of blastomeres at this early stage can result

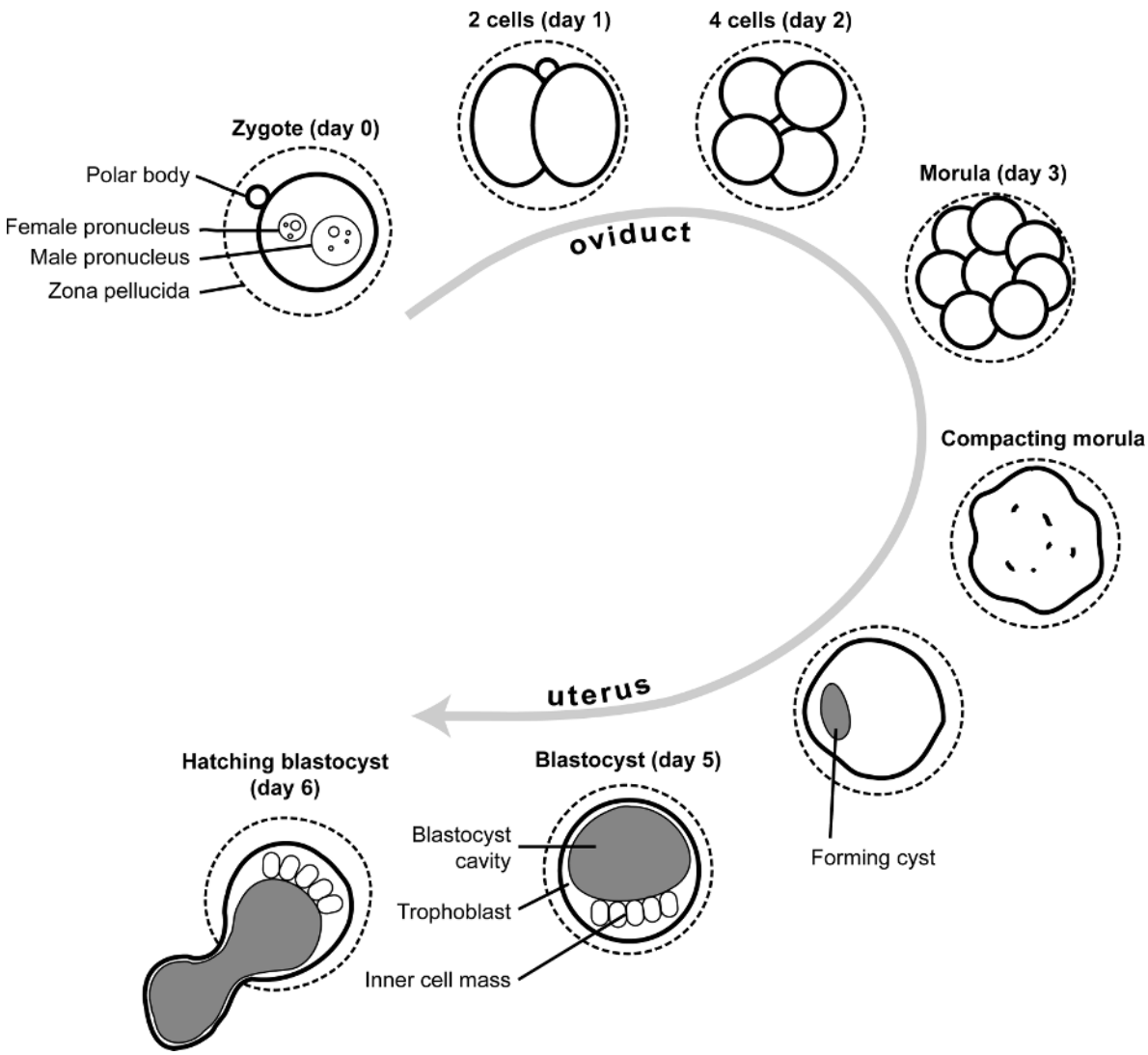

Figure 12.1 Preimplantation embryonic development. The zygote undergoes cleavage divisions as it travels down the oviduct towards the uterus. At roughly day 6, the blastocyst hatches from the zona pellucida in preparation for implantation.

in mono-zygotic twins, with the two fetuses having completely separate placenta.

Generally at the eight-cell stage, the morula undergoes a process called compaction, whereby the blastomeres start forming intercellular junctional complexes (*gap*, *adherens* and *tight* junctions), resulting in them becoming more closely apposed, making the surface of the morula smooth in appearance. This is accompanied by the blastomeres becoming polarized, with exposed apical membrane and embedded basolateral membranes acquiring different properties (Fig. 12.1).

As compaction proceeds, blastomeres lose their totipotency. External cells differentiate to form abundant gap and tight junctions, reorganize their cytoskeleton to flatten their shapes and increase adhesion with one another through E-cadherin. Inner cells remain apolar and round in shape. The process of compaction is completed by about the 32-cell stage, by which time the embryo starts to become a *blastocyst*, expanding due to the accumulation of fluid-filled spaces within it. These fluid-filled spaces coalesce to form an asymmetrically positioned blastocyst cavity. Inside cells remain round in shape, positioned on one side and become the pluripotent *inner cell mass* (ICM), while outside cells become the *trophoblast* (Fig. 12.1). The ICM gives rise ultimately to the fetus (as well as contributing to the placenta) while the trophoblast contributes exclusively to the placenta. The region of the trophoblast abutting the ICM is called *polar trophoblast*, while that lining the blastocyst cavity is called *mural trophoblast*. Monozygotic twins with a shared placenta (one chorion and two amnions or one chorion and one amnion) result from the ICM splitting after the blastocyst stage. Time-lapse movies of human embryo cleavage and blastocyst formation are available through the work of Mio and Maeda [3].

It is from the ICM that *embryonic stem cells* (ES cells) are derived. The ICM and trophoblast lineages are characterized by the expression of different genes. For example, ICM cells notably express pluripotent markers such as *NANOG* and zygotic *OCT4*, while trophoblast cells express *CDX2*.

Implantation and formation of the bilaminar embryonic disc

Fertilization takes place in the fallopian tube, after which the cleaving embryo travels towards the uterus. Approximately five days after fertilization, the embryo reaches the uterus. By that time it is a blastocyst, but is still enclosed by the zona pellucida. The zona pellucida is important in preventing the embryo from implanting prematurely in the oviduct, resulting in ectopic pregnancy. Once it reaches the uterus, the blastocyst 'hatches' out of the zona pellucida (Fig. 12.1). This is achieved by localized enzymatic digestion of the zona pellucida by the embryo, creating a hole through which the embryo can slip out. The embryo is now ready to undergo implantation, a process that begins on roughly day 6.

Around this time, the ICM differentiates into two cell layers, the *epiblast* (adjacent to the polar trophectoderm) and *hypoblast* (adjacent to the blastocyst cavity). As the embryo implants, rapidly dividing cells of the polar trophoblast lose their cell membrane and coalesce with one another to form the *syncytiotrophoblast*. In contrast, cells of the mural trophoblast remain as individual cells, forming the *cytotrophoblast* (Fig. 12.2). The syncytiotrophoblast facilitates implantation by secreting metalloproteases and collagenases to digest the extracellular matrix of the uterine endometrium. After implantation, the syncytiotrophoblast ultimately comes to envelop the entire embryo, which now consists of an outer layer of cytotrophoblast enclosing the epiblast, hypoblast and blastocyst cavity (Fig. 12.2).

In female embryos, *X chromosome inactivation* (XCI) occurs in cells of the epiblast. This is a process of *dosage compensation* that ensures both males and females produce the same amount of proteins encoded by the X chromosome, despite females having two (XX) and males only one X chromosome (XY) in each cell. The inactivated X chromosome condenses into heterochromatin and is called the *Barr body*. XCI is triggered by the expression of *XIST* transcript from the X chromosome to be silenced. Either the maternal or paternal X chromosome is randomly inactivated. As a result, the female fetus (and adult) is a *genetic mosaic*, composed of a mixture of cells expressing genes from the paternal or maternal X chromosome.

Around day 8, the *amniotic cavity* forms between the epiblast and overlying trophoblast. Cells from the epiblast spread out to line this cavity, ultimately forming the *amnion*. Cells from the hypoblast spread out along the cytotrophoblast, forming *Heuser's membrane*. This encloses the blastocyst cavity, which now comes to be called the *primary yolk sac* (Fig. 12.2). The epiblast and hypoblast separating the amniotic cavity from the primary yolk sac is now called the *bilaminar embryonic disc*.

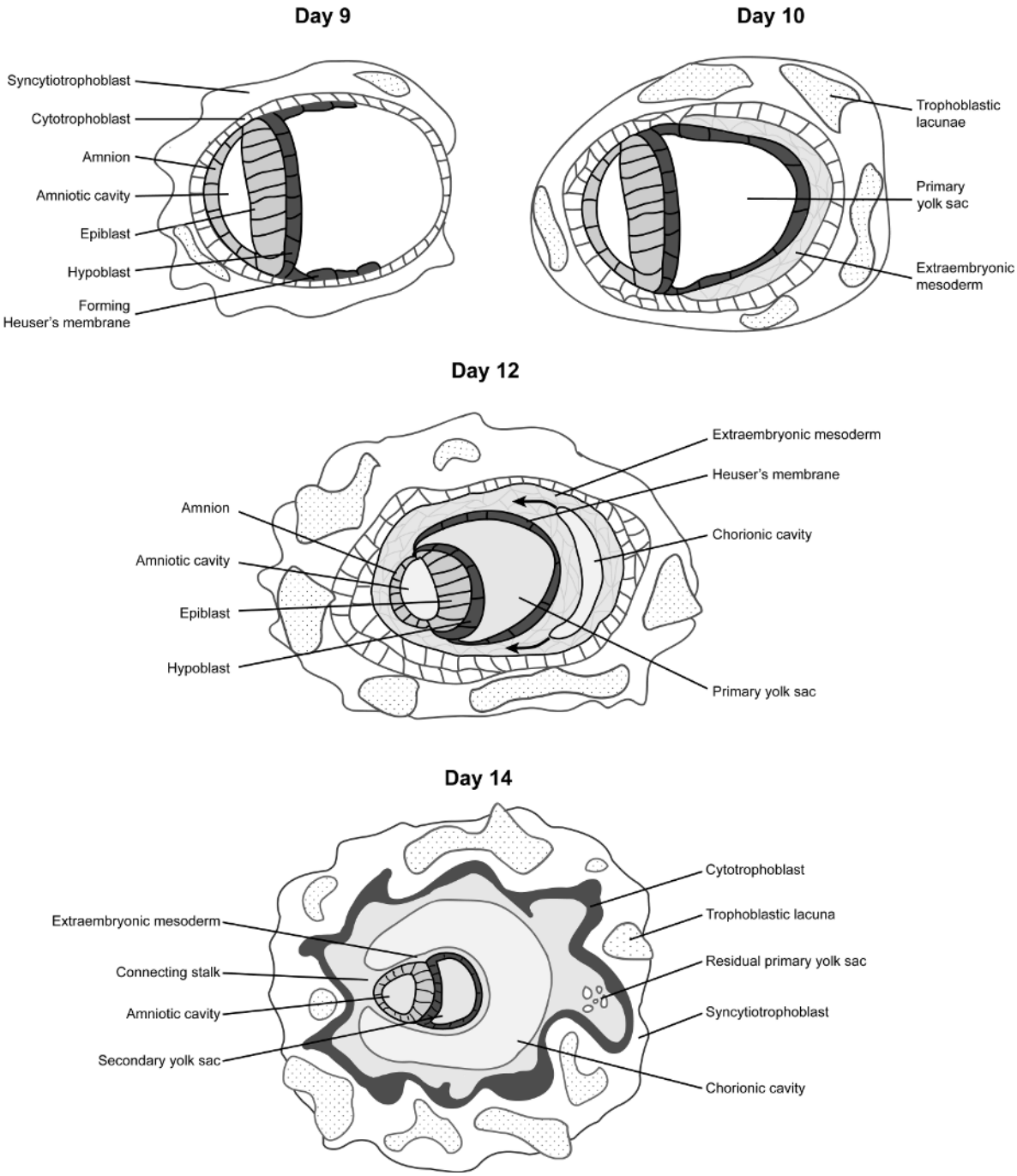

Figure 12.2 Formation of the bilaminar embryonic disc. By this stage, the embryo has implanted in the uterine endometrium (not shown). Heuser's membrane grows to enclose the primary yolk sac. At about day 12, the chorionic cavity forms as a space within the extraembryonic mesoderm and spreads to enclose the epiblast (arrows).

Extraembryonic mesoderm, thought to be derived from the hypoblast or primary yolk sac, forms between Heuser's membrane and the overlying cytotrophoblast, partially occluding the cavity of the primary yolk sac. It also grows over the amnion, resulting in the embryonic disc (along with the amniotic and yolk sac cavities) being embedded within a mass of extraembryonic mesoderm. As the embryo grows, a

new space emerges within the extraembryonic mesoderm – the *chorionic cavity*. This space gradually grows around the embryonic disc, until around the end of week 2; the embryonic disc is suspended within this cavity by a *connecting stalk* derived from extraembryonic mesoderm. While the chorionic cavity is expanding, portions of the primary yolk sac get pinched off, leaving behind a smaller *definitive* or *secondary yolk sac* (Fig. 12.2).

Extraembryonic structures and blood formation

Primitive *hematopoiesis* and *vasculogenesis* first occur in the yolk sac, leading to the production of blood and vessels respectively. *Blood islands*, clusters of primitive erythrocytes surrounded by endothelial cells, arise from the extraembryonic mesoderm in the yolk sac. These islands grow and connect with each other to form the vasculature (and blood) of the fetal part of the *uteroplacental* circulation.

Beginning day 9, vacuoles form within the syncytiotrophoblast and fuse with one another to form *trophoblastic lacunae* (Fig. 12.2). Maternal uterine capillaries in the near vicinity anastamose with these lacunae between the 11th and 13th days. As this process is ongoing, the cytotrophoblast grows into the syncytiotrophoblast to form elongated *villi*. Syncytiotrophoblast-covered villi protrude into the trophoblastic lacunae, forming *primary chorionic stem villi*. Around day 16, extraembryonic mesoderm invades their cores, transforming them into *secondary chorionic stem villi*. These in turn become *tertiary chorionic stem villi* upon formation of vasculature from the extraembryonic mesoderm. Thus maternal and fetal gas and nutrient exchange occurs between these villi and the trophoblastic lacunae.

Gastrulation and the formation of three germ layers

Gastrulation is a crucial process in embryogenesis. It results in: the generation of the three *primary germ layers* from which all fetal tissues are derived; the conversion of the bilaminar embryonic disc into a trilaminar disc; the establishment of the primary (rostrocaudal) body axis; and the organization of the basic body plan.

Around day 14 a midline thickening, the *primitive streak*, appears in the epiblast in the caudal region of the bilaminar embryonic disc. Epithelial epiblast cells within the primitive streak undergo an epithelial-to-mesenchymal transition, delaminate and ingress into the interior of the embryo to displace the underlying hypoblast (Fig. 12.3). These epiblast-derived cells re-epithelialize, forming the *definitive endoderm* that forms the inner lining of the gut tube as well as associated organs. Cells that ingress through the streak subsequent to the formation of the definitive endoderm (around day 16) form *mesoderm* (Fig. 12.3) that gives rise to most internal organs. Cells of the epiblast that do not ingress through the streak differentiate into *ectoderm* that gives rise to the central nervous system and epidermis of the skin. As a result of this process the bilaminar embryonic disc, consisting of

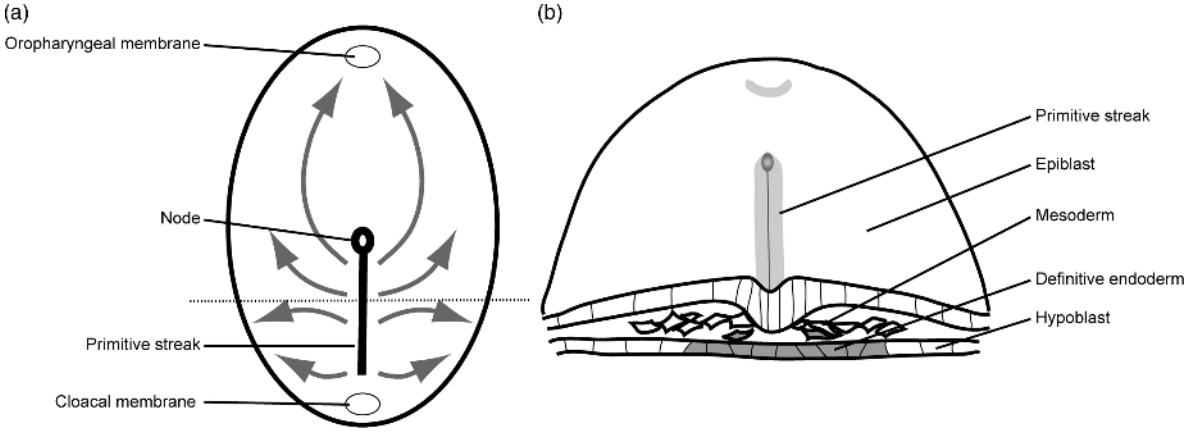

Figure 12.3 Gastrulation. Panel (a) shows the epiblast surface in a dorsal view of the embryonic disc. The arrows depict streams of mesodermal cells migrating beneath the epiblast. Panel (b) shows a section view along the dotted line in panel (a). Cells of the epiblast undergo an epithelial to mesenchymal transition and delaminate to form the mesoderm.

epiblast and hypoblast, is converted into a *trilaminar embryonic disc* consisting of ectoderm, endoderm and an intervening layer of mesoderm.

At the rostral end of the primitive streak is a specialized expanded structure called the *primitive node*. The node acts as an embryonic *organizer*, in that it is responsible for organizing surrounding tissues without itself contributing to them. The properties of embryonic organizers was demonstrated by Hilde Mangold and Hans Spemann in seminal experiments where they transplanted the structure analogous to the node from one amphibian embryo to another and showed that this resulted in the formation of a second body axis in the recipient embryo.

Towards the end of the third week, two depressions form in the ectoderm, at the rostral and caudal ends of the embryo disc. The ectoderm in the depressions fuses tightly with the endoderm beneath, excluding the mesoderm and forming bilaminar membranes in these regions. The rostral membrane is the *oropharyngeal* membrane which later degenerates to form the opening of the oral cavity. The caudal membrane is the *cloacal* membrane, which degenerates later to form the opening of the digestive, urinary and genital tracts.

The ingression of cells through the primitive streak continues for several days. This is accompanied by the regression of the streak towards the caudal end, so that as the embryo develops, the streak grows shorter. Thus, a rostrocaudal 'temporal gradient' of development exists, with rostral structures being more developmentally advanced than caudal structures.

Formation of the major body axes

The orientation of the primitive streak defines the rostrocaudal axis of the embryos. On the basis of experiments in chick and mouse embryos, it is understood that the hypoblast (or a subset of cells within it) is responsible for determining the position at which the primitive streak forms and consequently, the orientation of the rostrocaudal axis.

In one sense, the dorsoventral axis is determined already at the bilaminar disc stage, from the way the epiblast and hypoblast are arranged relative to one another. However, it is only after gastrulation and the formation of the notochord that there is overt patterning along the dorsoventral axis, manifest for example in the patterning of the neural tube in response to signals from the notochord and surface ectoderm.

The left-right axis is the last to be established. Though externally humans are bilaterally symmetrical, internally there is asymmetry in the position or structure of organs – the heart is on the left, the liver is mostly on the right, the two lungs have different numbers of lobes, the two kidneys are at different levels, etc. This normal asymmetry is referred to as *situs solitus* and is generated by the ventral cells of the node. These cells have characteristic cilia that rotate. As the cilia are tilted, their rotation creates a directional leftward *nodal flow*. This directional flow is understood to lead to the accumulation of *nodal vesicular parcels* containing SHH protein on the left margin of the node, which triggers increased levels of Ca^{2+} signalling on that side. This in turn leads to the asymmetric left-sided expression of genes like *NODAL*, *LEFTY1/2* and *PITX2* which results ultimately in the generation of morphological asymmetries. Disruption of this process can result in situs defects, such as *situs inversus*, where the handedness of visceral organs is reversed.

Neurulation

Around day 18, the node induces the ectoderm immediately rostral to it to change shape. These cells elongate to become pseudostratified and the area thickens to become the *neural plate*, composed of *neuroectoderm*. Thus as the node regresses towards the posterior, neural induction follows in its wake. As the streak regresses, it lays down a rod-like mesodermal structure in the midline, the *notochord*, formed from cells that ingress through the node. The notochord induces the overlying neural plate to fold in and eventually pinch off to form the *neural tube*. The neural tube gives rise to the central nervous system. The notochord and surface ectoderm play central roles in the dorso-ventral patterning of the neural tube. A gradient of *SHH* from the notochord acts in combination with a gradient of BMPs from the surface ectoderm to specify neuronal subtypes in different regions of the neural tube.

The neural plate folds into a tube by elevating its edges toward the midline by the movement of surface ectoderm. In a process called *neural tube closure*, the edges form medial hinge points, meet medially and fuse to pinch off the neural tube, which sinks below the surface ectoderm. Closure defects can result in severe congenital abnormalities such as *craniorachischisis* or *spina bifida*.

Neural crest cells are migratory mesenchymal cells originating from the crest of the neural fold as it closes.

They are internalized by the ectoderm when the neural tube is pinched off. Neural crest cells contribute to a very wide range of tissues and organs. For example, they give rise to neurons and glia of the peripheral nervous system, the cartilage and bones of the face, melanocytes and contribute to the septation of the developing heart.

Endodermal and mesodermal derivatives

The endoderm forms the embryonic gut tube, which gives rise to the inner lining of the digestive tract. It also contributes to organs that arise from evaginations of the embryonic gut tube, such as the lung, liver and pancreas. The thyroid, thymus and parathyroid glands are also derived from the endodermal cells of the *pharyngeal pouches*.

The cells to emerge from the rostral third of the primitive streak form an arc of *cardiogenic* mesoderm rostral to the primitive streak called the *cardiac crescent*. This mesoderm gives rise to the primitive heart tube and much of the adult heart, particularly the left ventricle and the two atria. The remaining mesoderm can be subdivided into *axial*, *paraxial*, *intermediate* and *lateral-plate* mesoderm on the basis of position from the centre of the embryo. Axial mesoderm lies in the midline and forms the notochord. Paraxial mesoderm lies to either side of the neural tube. In the head region it forms the head mesoderm which gives rise to striated muscles of the head, jaw and neck regions. In the trunk, the paraxial mesoderm condenses into paired segmental blocks called *somites*. Somites are a manifestation of the segmental architecture of the basic mammalian body plan. Each somite gives rise to a *sclerotome, myotome* and *dermatome*, which form bone, muscle and the dermis of skin respectively. Somites form and develop in rostrocaudal progression, so in the more rostral region somites differentiate into bone and muscle while at the same time in more caudal regions, they are just being formed from the paraxial mesoderm.

Intermediate mesoderm lies just lateral to paraxial mesoderm. It gives rise to the *pronephros*, *mesonephros* and *metanephros* in rostrocaudal succession. The metanephros form the definitive kidneys. Intermediate mesoderm also gives rise to the gonads and associated ducts.

Lateral plate mesoderm is the most peripheral mesoderm and gives rise to vasculature, lymphatic system, blood cells and the covering of visceral organs.

Germ cells

Early in gastrulation, a group of cells called *primordial germ cells* (*PGC*) are set aside to ultimately produce the gametes that give rise to the next generation. They are derived from the epiblast, but migrate to reside in the yolk sac. Later, between the fourth and sixth weeks, they migrate from the yolk sac through the wall of the gut tube, to the dorsal body wall. Here they populate a region of intermediate mesoderm that they induce to form paired *genital ridges* from which the gonads form.

Embryo folding

The final human body plan is one of a tube within a tube with the inner gut tube enclosed by an outer tube of ectoderm. However, as described, the embryo starts out as a flat disc, initially bilaminar, and then after gastrulation, trilaminar. *Embryonic folding* refers to the process by which this flat disc is converted to the final tubular form. This is achieved primarily through differential rates of growth in different regions of the embryo.

During the fourth week, the embryo and overlying amnion undergo rapid growth which, in combination with *convergent extension* movements of cells along the embryonic midline, result in the elongation of the body. The yolk sac in contrast does not grow, resulting in the margins of the embryonic disc being pulled down towards it (Fig. 12.4). Axial structures like the notochord and neural tube keep the dorsal midline of the embryo relatively rigid, causing the lateral margins to account for most of the folding movement. The cranial and caudal ends of the embryonic disc also fold in. Cranial, caudal and lateral folding movements result in the endoderm being enveloped, pinching it off internally to form the gut tube. The cranial and caudal ends of the gut tube are initially blind ending. The mouth and anus are formed later, by apoptosis of cells of the oropharyngeal and cloacal membranes respectively.

In addition to enclosing the endoderm to form the gut tube, embryonic folding movement also encloses mesoderm, which comes to line the gut tube and the body wall. In between these two layers of mesoderm is a space that forms the *intraembryonic coelom*. A wedge of mesoderm called the *septum transversum* grows across the coelom, contributing to the formation of the diaphragm and separating the coelom into abdominal and thoracic cavities. Cranial folding also brings the primitive heart tube, which is initially

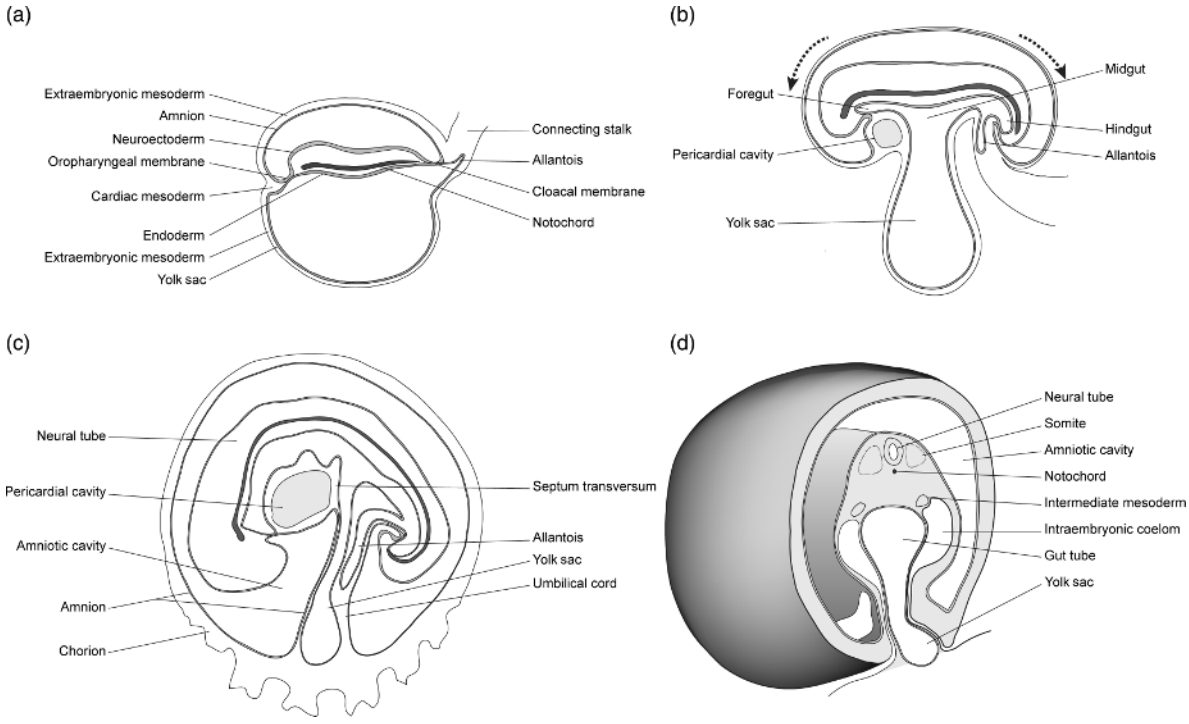

Figure 12.4 Embryonic folding. Transverse (panels a, b and c) and cross-sectional views (panel d) of different stages of embryonic folding. The diagrams are not to scale.

rostral to the developing brain, to its more caudal thoracic position.

The folds meet ventrally and fuse, forming the ventral body wall. This fusion occurs first in the cranial and caudal ends, with the yolk sac cavity in between, communicating directly with the gut tube. The regions of ventral fusion gradually move towards each other, constricting the yolk sac into a narrow stalk, still connected to the midgut. Early in the fourth week, a small diverticulum called the *allantois* (proximal regions of which give rise to the urinary bladder) emerges from the forming hindgut and grows into the connecting stalk. The yolk sac also comes to be pressed against the connecting stalk, which ultimately comes to be enclosed within the growing amniotic sac, to give the umbilical cord (Fig. 12.4).

These changes transform a sheet-like embryo into a tube, with the basic tissues roughly in place to undergo organogenesis.

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Chapter 13

Human organogenesis

Autumn Rowan-Hull

How a single cell, following gastrulation, can form into the correct tissue in the correct location, and how these tissues interact to form organs that make up our body, is a truly remarkable feat. As this achievement is obviously a huge area to cover, the goal of this chapter is to provide a framework which will enable students to gain a broad understanding of human organogenesis and encourage further investigation [1–3].

The previous chapter, 'Early embryogenesis', describes how the basic body plan of the embryo is laid out. The process of gastrulation creates the trilaminar embryo with its three germ layers: ectoderm, mesoderm and endoderm. It is these germ layers that contribute to the formation of each organ which makes up the embryo proper.

Musculoskeletal system

Muscles

Muscles are formed from the mesoderm. The early mesoderm condenses into distinct regions: prechordal, axial, paraxial, intermediate and lateral plate mesoderm (Fig. 13.1a). The paraxial mesoderm gives rise to muscles. The paraxial mesoderm first condenses into somitomeres and progressively extends rostrocaudally in pairs. These pairs then epithelialize into somites which, in time, run down the entire length of the embryonic body (Fig. 13.1b). In total, 42–44 pairs of somitomeres are created. The most caudal 6–8 pairs degenerate, while the first seven cranial pairs will remain as somitomeres and contribute to the head formation.

The trunk somites are further differentiated into two regions: the sclerotome and the dermamyotome (Fig. 13.1c). The sclerotome forms bones of the axial skeleton, while the dermamyotome further differentiates into the dermatome and myotome (Fig. 13.1d). The myotome gives rise to all the skeletal muscles of the body, head and limbs. The dorsomedial myotome cells proliferate to form epaxial myotome, which gives rise to back muscles. The dorsolateral cells form the hypaxial myotome which gives rise to muscles of the limbs and body wall (Fig. 13.1e). The other two muscle types, the cardiac and smooth muscle, are formed from the lateral plate mesoderm, as is most of the connective tissue with the exception of some head connective tissue which is derived from neural crest cells.

Myotome cells are committed to their fate in response to signals from the neural tube. The cells must migrate some distance to reach their target destination. Myogenic determination factors are a collective group of genes that have been shown to play a role in this commitment pathway [4]. Once the myogenic cells reach their chosen position within the embryo, they condense and reaggregate into a pre-muscle mass. It is only once they have arrived at their final destination that they begin to differentiate.

Myotomes differentiate into myoblasts which fuse to form multinucleated myotubes at week five. Innervation of the motor axons also begins at this time (Fig. 13.1f). Proteins are then expressed in these myoblasts that make up the contractile elements, and different fibre types are established. The connective tissue surrounding each forming muscle provides the cues for this differentiation. During the fetal period, the myoblasts continue to incorporate into myotubes, followed by myotube maturation into long muscle fibres. Sensory nerve processes enter muscle masses shortly after they have been established.

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

Skeletal system

Most of the cartilage and bones are also derived from mesoderm. Different subdivisions of the mesoderm give rise to the cartilage and bones of different regions of the body: the trunk, the limbs and the head. The axial skeleton, which runs along the midline, longitudinal axis of the body, includes the vertebrae and ribs and is derived from the sclerotome region of the somites (Fig. 13.1c–e). The cartilage and bones of the limbs are formed from the somatic lateral plate mesoderm (Fig. 13.1f). The skull, on the other hand, is derived from many sources of mesoderm tissue. Somitic sclerotomes and the most cranial somitomeres, as well as lateral plate mesoderm and neural crest, all contribute to cartilage and bones of the skull.

At the beginning of the fourth week, sclerotome, somitomeres, lateral plate mesoderm and neural crest cells become polymorphous and form mesenchymal tissue. This mesenchymal tissue is defined by its loosely organized morphology and its ability to migrate. These cells travel to their correct destination to become fibroblasts, chondroblasts (which form cartilage) or osteoblasts (bone-forming cells).

In most bones, mesenchymal cells condense and first give rise to hyaline cartilage, by a process called endochondral ossification. These cartilage cells die and leave spaces into which osteoblasts and blood vessels penetrate. As the osteoblasts begin to differentiate into osteocytes, they secrete a calcified extracellular matrix. Although all long bones are ossified at birth, this process continues for many years. A number of important bones are also formed by intramembranous ossification. During this process, bones are formed directly within the mesenchymal condensation where they form membranous sheets that directly secrete bone matrix.

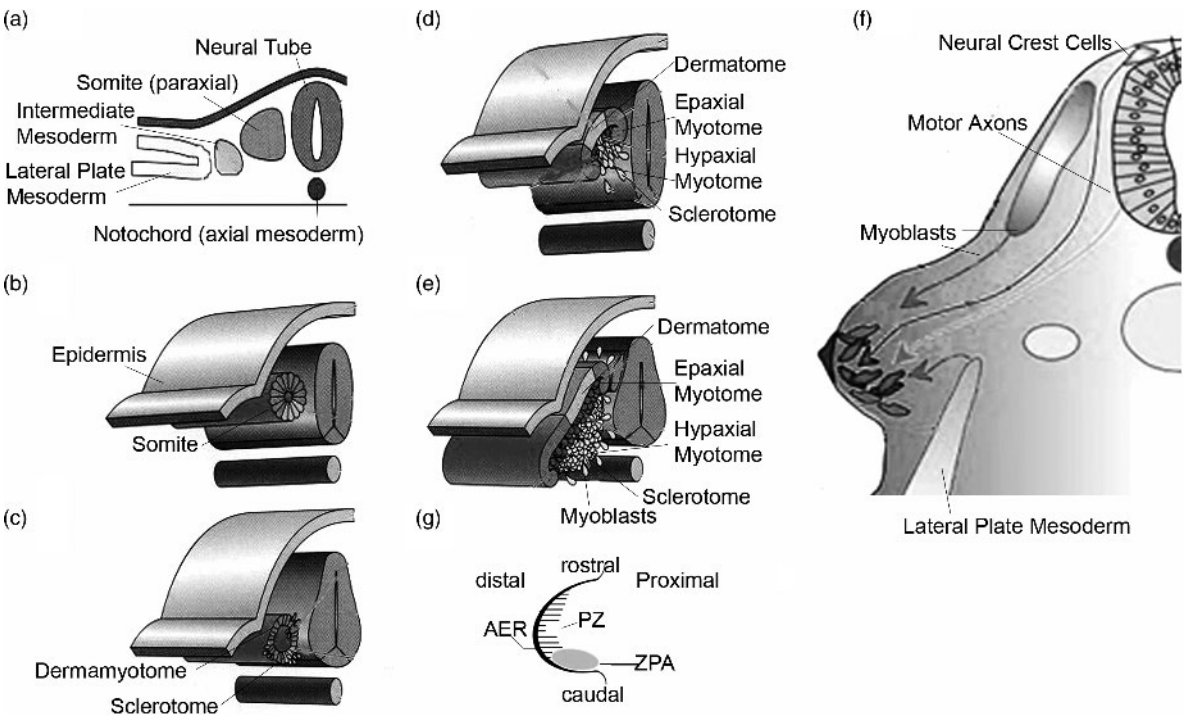

Figure 13.1 Development of the musculoskeletal system. (a) A transverse section of a 4-week-old embryo illustrating the different mesoderm regions surrounding the central neural tube. (b) The somatic cells condense in a rostrocaudal sequence. (c) Somites differentiate into sclerotome (cartilage and bone components) and dermamyotome (muscle and dermis of skin components). (d) The sclerotome cells migrate towards the neural tube. The lateral dermamyotome further differentiates into hypoaxial myotome, while the medial dermamyotome form epaxial myotome. (e) Muscle precursors, myoblasts, then form. (f) Limbs develop as buds from the lateral body wall, myoblasts, motor axons, lateral plate mesoderm and neural crest cells all contribute to limb formation. (g) The AER is formed at the tip of the limb bud while the underlying mesoderm forms the PZ. The ZPA is located in the caudal region of the limb bud. These regions are dependant on each other for patterning and growth of the limb. (Figure modified from Gilbert figs. 10–14 [2].)

Limb development

Limb development has been an intense area of investigation over the past two centuries because it has provided a framework used to analyze vertebrate development and pattern formation in the embryo as a whole [5]. The limb is also accessible and dispensable and analysis of a wide range of limb defects has allowed researchers to understand genetic contributions as well as environmental factors involved in birth defects.

The upper limb first develops as a bud from the lateral body wall of the embryo at levels C5 to T1 in the fourth week of development. The lower limb lags slightly behind and can be seen one to two days later at the level of L1 to L5. Hox genes, which are expressed at different levels along the rostrocaudal axis, appear to be responsible for allocating these so-called limb fields.

Limb bud formation begins with the activation of lateral plate mesoderm which starts to produce Fibroblast Growth Factor (FGF10). It is thought that FGF10 induces FGF8 in the surface epithelium to form the apical ectodermal ridge (AER) during week 5. Interactions between the AER and underlying mesoderm, now called the progress zone (PZ), allow cells to proliferate, which results in outward growth along the proximal-distal axis (body to finger or toes) (Fig. 13.1g). In one model, it is proposed that as the limb grows, cells leave the progress zone and differentiate into cartilage and muscle. The amount of time each cell spends in the progress zone determines which part of the limb will be formed. It is these cells that may also direct cells from the somites to migrate and form the skeletal muscle.

Limbs also require information to direct differentiation and growth along the rostrocaudal axis (i.e. thumb or big toe to little finger or toe) as well as the dorsoventral axis (i.e. palm or sole of foot to back of hand or top of foot). Patterning of the rostrocaudal axis is regulated by the zone of polarizing activity (ZPA). The ZPA is a cluster of cells located at the caudal border of the limb bud near the flank. FGF8 in the mesoderm induces the expression of both Retinoic Acid (Vitamin A) as well as Sonic Hedgehog (SHH) in the ZPA. These genes within the ZPA activate other gene networks to initiate rostrocaudal differentiation. Unlike the other axes, the ectoderm regulates the dorsoventral polarity of limb mesenchymal cells. The molecular signalling of Wnt7A is expressed in only the dorsal region of the ectoderm. Wnt7A causes the expression of the transcription factor Lmx1 which is instrumental in dorsalizing cells.

By six weeks, the hand and foot plates at the tip of the limb buds are visible. Finger and toe rays form by weeks 7 and 8. Programmed cell death creates the spaces between the digits. Limbs need to rotate along their long axis within the body to establish their correct orientation. The arm rotates 90° dorsolaterally along the long axis, allowing the elbow to point backwards. The leg rotates 90° ventromedially, positioning the knees forward. This happens during weeks 6–8. Limbs also need to be vascularized and innervated. Limb muscles are initially vascularized by an axial artery which develops along the central axis of the limb. In the upper limb, this artery joins the fifth lumbar intersegmental artery, while the axial artery forms the subclavian, axillary and branchial arteries. In the lower limb, the axial artery joins the fifth lumbar intersegmental artery. The axial artery degenerates and a new branch from the fifth lumbar arises as the external iliac, which supplies most of the leg. Innervation of the limbs is from the ventral rami of spinal nerve located at vertebral level C5–T1 in the upper limb and L2–L3 in the lower limb. Upon reaching the base of the limb, a plexus is formed and nerves establish motor synapses within the developing skeletal muscles. Sensory nerves follow the motor nerves within the limb and use them as guides.

Clinical corner

Limb defects are relatively common and are often found as a component of a more serious congenital syndrome. Most limb defects are either a reduction in part of the limb (loss of part of a limb), a duplication defect (extra digits) or dysplasia (abnormal tissue amounts). The major factors causing these abnormalities are: genetic in origin (e.g. Apert syndrome); environmental (e.g. Fetal Alcohol Syndrome, Thalidomide) or result from the environment within the uterus (e.g. an amniotic band).

The nervous system

The nervous system can be divided broadly into the central and the peripheral nervous system. The nervous system contains various different types of nerve cells and supporting cells [6]. Remarkably, almost all of this complex nervous system can be derived from a single germ layer, the embryonic ectoderm.

Early neural development

The process of neurulation begins very early in the third week of embryonic development (Fig. 13.2a).

Signals from the node, or organizer, induce the surrounding ectoderm to become the neural plate. This interaction requires that the ectoderm cells are competent to respond to neural-inducing signals. These signals emanate from the node in a planar fashion. Vertical signals emanating from the notochord then reinforce this interaction. The neural plate thickens and lengthens as the node regresses. Laterally, the plate edges thicken to form the neural fold and these folds turn inwards, forming the neural groove along the midline (Fig. 13.2b). The edges continue to fold in at the cranial end, to form the start of the neural tube, at about 21 days (Fig. 2c). The neural tube develops progressively in a rostral to caudal direction leaving the anterior and posterior neuropore open at each end. These eventually close at the middle and end of the fourth week, respectively. Before the two sides of the neural tube fuse, cells located at the two edges of the neural plate migrate away from the edges. These are the neural crest cells (Fig. 13.2d). The most rostral end of the neural tube forms the brain while the remainder of the tube forms the spinal cord.

Cytodifferentiation

Differentiation of neuroepithelial cells into neurons, glia and epididymal cells of the central nervous system begins in the fourth week in the rhombencephalic

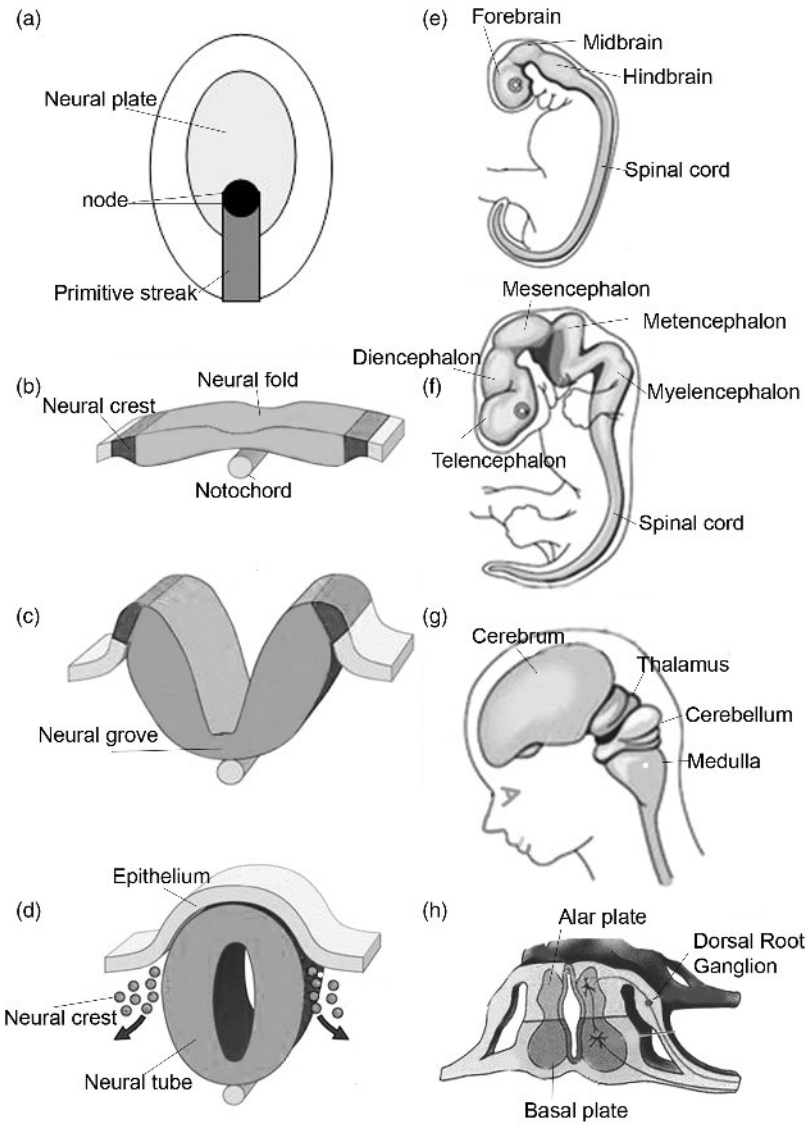

Figure 13.2 Development of the nervous system. (a) Dorsal view of epiblast which shows the position of the node and induced neural plate in the second week of development. (b) Transverse section of an embryo in the second week of development. (c) The neural plate folds to form the neural tube (d). Neural crest cells then migrate away from the neural tube. (e) The nervous system develops into the three vesicles of the brain plus the spinal cord in the fourth week. (f) Five-vesicle stage of the brain plus spinal cord in a 6-week-old embryo. (g) The developing brain at 11 weeks. (h) Developing spinal cord in a 6-week-old embryo.

region and proceeds rostrally and caudally as the neural tube closes. Neuroblasts are born in the ventricular zone that surrounds the central lumen. They then migrate towards the outer surface of the neural tube to form the mantle zone. These cells give rise to neurons of the grey matter. Neuronal axons extending peripherally from the mantle layer form the marginal zone, which later forms the white matter. Glioblasts are also formed in the ventricular layer which differentiates into two types of astrocytes: radial glial cells and some oligodendrocytes. Glia provide metabolic and structural support to the neurons in the central nervous system. The cells that remain in the lumen differentiate into epididymal cells responsible for secretion and absorption of cerebrospinal fluid. The generation of neurons, however, in areas of the brain that develop a cortex, including the cerebellum and cerebral hemispheres, is more complex.

Development of the brain

The subdivisions in the brain are first apparent by a fold, the mesencephalic flexure, appearing in the neural plate. Other indentations in the neural fold demark the three major divisions of the brain: the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain) (Fig. 13.2e). The pontine flexure also appears in the hindbrain of the neural tube; while another bend in the neural tube occurs at the junction between the brain and spinal cord and is called the cervical flexure.

From this point the three primary vesicles of the brain further divide in the fifth week, into five secondary vesicles (Fig. 13.2f). The prosencephalon divides into the telencephalon and diencephalon. The telencephalon, located at the most rostral end, goes on to form the cerebrum or cerebral hemispheres. The diencephalon, which includes the optic placodes, forms the thalamus, hypothalamus and pituitary. The mesencephalon does not undergo further division but forms the colliculi amongst other structures. The rhombencephalon divides into the metencephalon, which gives rise to the pons and cerebellum, and the myelencephalon, which forms the medulla oblongata (Fig. 13.2g). Specific combinations of gene expression and signalling centres direct the unique development of each region.

Development of the spinal cord

The spinal cord is continuous with the myelencephalon. The mantle zone develops within the differentiating neuroblast to form a thickening in the dorsal and ventral regions of the cord called the alar and basal plates, respectively (Fig. 13.2h). The alar plate (dorsal horn) becomes the sensory region. Axons from the sensory bipolar neurons located in the dorsal root ganglia enter into the dorsal horn and synapse with neuronal cell bodies. In contrast, the basal plate (ventral horn) becomes the motor region of the spinal cord where developing axons exit the spinal cord, forming the ventral root of the spinal nerves, and innervate voluntary muscles. As both horns develop, the dorsal and ventral neuroepithelium thins in the midline which forms the roof and floor plate that go on to serve as commissures for nerve fibres. In addition, intermediate or lateral horns develop at spinal levels T1–L3 and S2–S4. These neurons project their axons out of the spinal cord via the ventral root. These cells differentiate into the sympathetic and sacral parasympathetic preganglionic neurons.

Development of the peripheral nervous system

The peripheral nervous system (PNS) is subdivided into two: (a) the somatic or sensory nervous system, which innervates the voluntary muscles of the body and transmits signals to the CNS; (b) the autonomic nervous system which is strictly motor and controls most of the involuntary activities of the body. The autonomic nervous system is further divided into the parasympathetic nervous system, which is concerned with peace and relaxation, and the sympathetic nervous system, which controls the fight or flight response. The gut also has its own neural control called the enteric nervous system. Neural crest cells form most of the PNS, although some come from the ectodermal placodes located in ectodermal regions of the head and neck. Support cells are also derived from the neural crest derived and include satellite and Schwann cells. Other support cells such as connective tissue fibroblasts are derived from the lateral plate mesoderm.

Clinical corner

Neural tube defects such as spina bifida can occur when the neural tube fails to close properly. It can also result when two halves of the vertebral arch fail to fuse, usually occurring at the base of the spine.

Development of the head, neck, eyes and ears

The head region of the embryo is formed from the pharyngeal arches that develop in the fourth week from mesenchymal cells derived from the neural crest, lateral plate mesoderm and paraxial regions.

There are five pairs of pharyngeal arches that form in a rostrocaudal succession, numbered 1, 2, 3, 4 and 6. Arch 5 never develops in humans or degrades quickly. Each arch consists of an ectodermal surface (pharyngeal clefts or grooves), mesenchymal core and endodermal inner surface (pharyngeal pouch and membrane) and contains cartilaginous skeletal elements, striated muscles, a cranial nerve and a aortic artery.

Arch 1 forms the upper and lower jaw and also participates in face and palate development. Arch 2 forms structures that support the jaw as well as contribute to the ear and hyoid. The third arch also contributes to the hyoid bone and greater cornu, while 4 and 6 fuse to contribute to laryngeal development. The pharyngeal pouches grow as pockets between arches. The first pouch lies between the first and second arches and gives rise largely to components of the ear. The second pouch is mostly concerned with palatine tonsil development. The third contributes to parathyroid and thymus glands, while the fourth also plays a part in parathyroid and thyroid development. The fifth pouch becomes fused with part of the fourth pouch.

The human face is formed between the fourth and tenth weeks, by fusion of five facial primordia which includes the frountonasal prominence, and a pair of maxillary and mandibular prominences, which grow and merge together. A pair of ectodermal placodes develops on the frountonasal prominence which also gives rise to the nose. Both parts of the eye and ear are formed from ectodermal placodes.

Clinical corner

A cleft lip can be caused when the two maxillary prominences do not merge with each other properly.

Cardiovascular system

The cardiovascular system is one of the first systems in the embryo to develop [7]. This is necessary in order to transport oxygen and nutrients to the cells in the rapidly growing embryo because it is no longer possible to do this by diffusion.

In the middle of the third week of embryonic development, angiogenic clusters start to form in the mesoderm in the rostral end of the embryo (Fig. 13.3a). These clusters aggregate to form blood islands in a horseshoe-shaped cardiogenic region. The blood islands consist of haemoblasts which generate all blood cells and endothelial cells which form the vessel walls. Proliferation and fusion of these blood islands creates a primitive network of capillaries.

The embryo then undergoes a series of folding events. The rostral end of the embryo folds cranially so the heart ends up in the future thorax region, while lateral folding brings the cardiac region together in the midline. The endothelial heart tubes then fuse to form a single primitive heart tube with a cranial and caudal end. The primitive heart tube is divided into a number of primitive chambers separated by grooves (Fig. 13.3b). In a caudal to rostral direction the chambers are: (a) the sinus venosus consisting of right and left horns, which gives rise to the right atria and vein; (b) primitive atria which develop into some of the right atrium and all of the left atrium; (c) primitive ventricle that will form the left ventricle; (d) the bulbus cordis, which gives rise to the right ventricle and some outflow tracts; and (e) the paired dorsal aortae.

The primitive heart begins to pump even before the heart tubes fuse together, and by day 22 contractions begin in the sinus venosus and move through the heart in peristaltic-like waves. Mesenchyme surrounding the heart tube thickens to form a myoepicardial mantle, which is contractile and later gives rise to the myocardium, forming the muscular wall and the epicardium or visceral pericardium, covering the outside of the tube. The endocardinal tube, which later becomes the endothelial lining of the heart, is separated from this myoepicardial mantle by gelatinous connective tissue called cardiac jelly.

During the third week, the heart undergoes a series of looping movements, which changes the shape of the heart allowing the four presumptive chambers of the heart to be brought into their definitive position (Fig. 13.3c–e). This obviously has a profound effect on the direction of blood flow through the heart tube and is also the first morphological sign of left/right asymmetry in the embryo. This looping is made possible by the breaking down of the dorsal mesocardium, which suspends the developing heart from the dorsal body wall.

Development of the U-shaped bulboventricular loop is formed through differential growth whereby the atrium and sinus venosus come to lie dorsal to the bulbus cordis and ventricle. The heart then bends laterally to the right (dextral looping) to form the

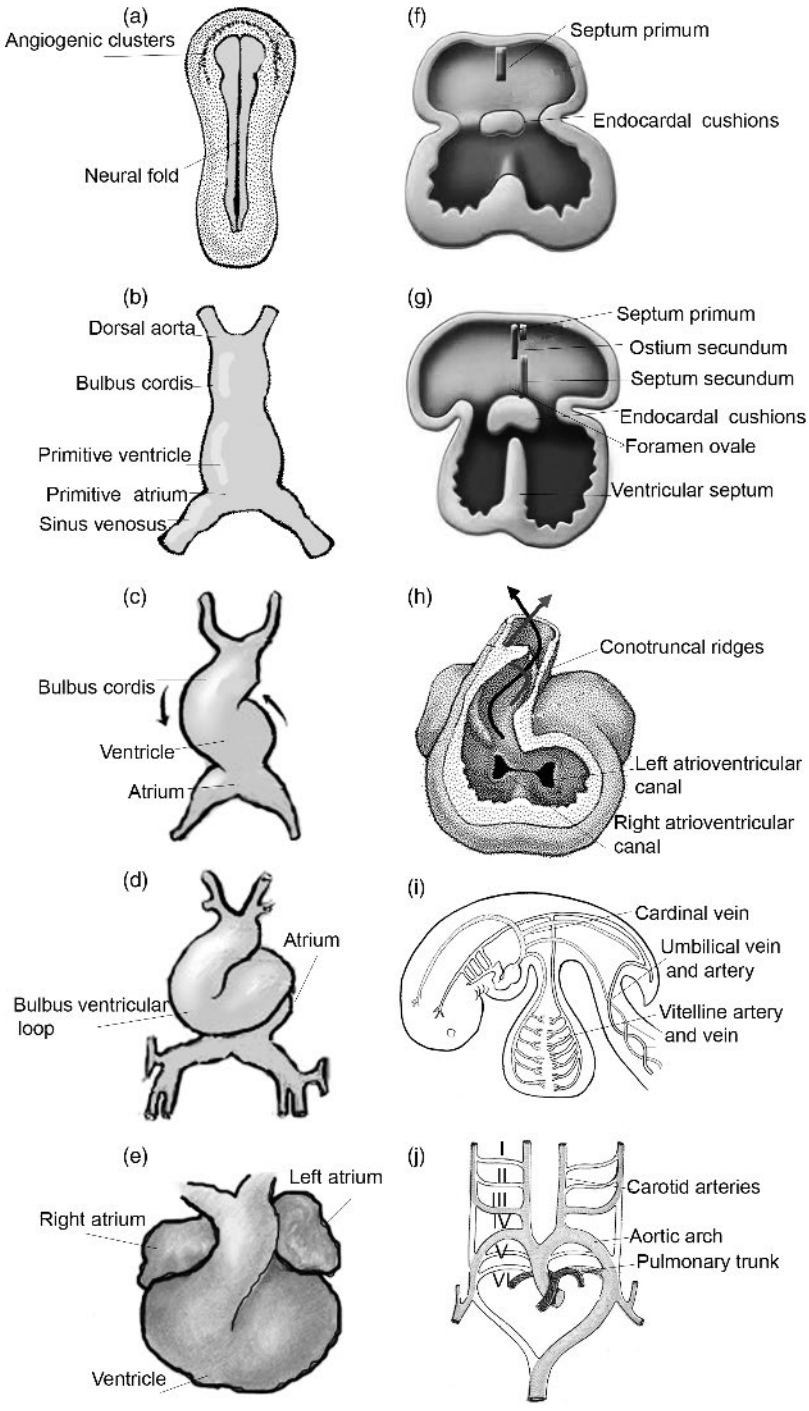

Figure 13.3 Cardiovascular system. (a) Angiogenic clusters form in the mesoderm in a horseshoe-shaped area in the rostral end of the embryo in the third week of development. (b) By 22 days the embryo has formed a primitive heart tube. The primitive heart tube then undergoes dextral looping between 23 days (c) and 26 days (d). (e) At 30–35 days looping is complete and the four primitive heart chambers can be distinguished from each other. Septation then begins. (f) The atria first starts to divide into two chambers by the growth of the septum primum from the roof of the atrial chamber during the fifth week of development. (g) During the sixth week of development the more muscular septum secundum then grows down towards the developing endocardial cushions but never fuses with the cushions. Perforations are made in the septum primum called the ostium secundum to allow blood to flow from the right ventral to the left ventrical via the foramen ovale. The ventricular septum begins to separate the ventricles. (h) Starting in the fifth week conotruncal ridges begin to swell and develop as a spiral structure eventually separating the pulmonary truck and aorta. The membranous interventricular septum fuses with the muscular interventricular septum creating a left and right ventricle. (i) In the fifth week of development the venous system of three major sets of veins: the vitelline, umbilical and cardinal veins. (j) The arterial system develops first as a series of aortic arches which are subsequently remodelled.

S-shaped heart tube allowing the atria to lie above the bulbus cordis. The basic, but internally unsegmented, heart shape is achieved by four and a half weeks. The heart is then organized further to achieve: (a) septation of the common atrium into left and right, (b) septation of the common atrioventricular canal, (c) division of the outflow tract, and (d) septation of the ventricle into left and right.

Septation of the common atrium

The sinus horns are incorporated into the right posterior wall of the primitive atrium, as the smooth walled sinus venarum, and continue to expand to give rise to the definitive atrium. A single pulmonary vein sprouts from the left atrium which branches and grows towards the lungs. In the atria, a sheet of crescent-shaped tissue, the septum primum, grows down from the common roof (Fig. 13.3f). This extends towards endocardinal cushions that start to enlarge and divide the atrioventral canal. At this time programmed cell death creates perforations in the top of the septum primum to form a hole called the ostium secundum. A second shaped ridge, the thick muscular septum secundum, starts to grow down on the right of the septum primum (Fig. 13.3g). This tissue, however, does not fuse with the septum intermedium. The gap left by the septum secundum is called the foramen ovale and acts as a right-left interatrial shunt, passing blood that is rich in oxygen and nutrients from the placenta.

Division of the atrioventral canal

At the end of the fourth week, within the inferior and superior walls of the heart, two mesenchymal masses, called the endocardinal cushions, develop. These masses grow towards each other and by the end of the fifth week, fuse forming two separate passages between the atria and ventricle. A pair of lateral cushions also forms.

Atrioventricular valves

The valves which form between the fifth and eighth weeks are made by sculpting the heart wall through cell death. The spaces left by the dead cells result in the formation of the chordae tendinae which support the heart valves. Outgrowth of the vessel walls forms both the pulmonary and aortic valves.

Septation of the ventricles

The blood flow through the heart becomes separated into two streams. First, blood from the placenta, high in nutrients and oxygen, enters the right atrium via the inferior vena cava and flows through the interatrial shunt into the left atrium. It then flows into the left side of the ventricle before exiting. In contrast, blood returning from the embryo, lower in nutrients and oxygen, enters the right atrium via the superior vena cava and flows into the right side of the ventricle and out. Both blood flows leave the truncus arteriosus but spiral around each other, maintaining separation. The force of the blood flow starts to hollow out the right and left ventricles, leaving the muscular interventricular septum (Fig. 13.3g). However, this only partially separates the ventricles. Haemodynamic forces, caused by the two spiralling blood streams, act on the cardiac-rich wall of the outflow tract. This pressure causes formation of spiral conotruncal ridges, which fuse together, thus dividing the outflow tract (Fig. 13.3h). Fusion of the ridges creates separate aortic and pulmonary outflow tracts. By the end of seventh week, conotruncal ridges fuse with the muscular interventricular septum forming the membranous interventricular septum, finally separating the ventricles.

Venous system

There are three paired veins that drain into the heart at week 4: the vitelline veins, the umbilical veins and the common cardinal vein (Fig. 13.3i). The vitelline vein follows the yolk sac into the embryo and enters the sinus venosus after passing through the septum transversum. At the same time the endothelial primordium of the liver grows into the septum transversum. The venous system changes in the lower body because of the effect of the growing liver which surrounds the vitelline and umbilical vein. The ductus venosus develops within the liver, forcing blood carried by the left umbilical vein, which is high in oxygen, through the liver. This then drains into the inferior vena cava to enter the right atrium via the right sinus horn. The right umbilical vein then regresses. The venous system also changes in the upper body. The anterior cardinal vein develops into paired jugular veins, and a new vessel called the left branchiocephalic vein forms which channels blood from the left upper body into the right jugular and then into the superior vena cava which drains into the right atrium.

Arterial system

Pairs of branchial arches form during the fourth and fifth weeks (Fig. 13.3j). Aortic arches arising from the aortic sac grow into these branchial arches. The aortic arches terminate in paired dorsal aorta that eventually fuse to form a single aorta lying caudal to the branchial arches. In total, five pairs of aortic arches will form (I, II, III, IV, VI) but they are not all present at once. It is believed that the aortic arch system is an evolutionary remnant. The aortic arch system starts to remodel to form the separate aortic and pulmonary trunks at the end of the fourth week. This is achieved

by aortic arches I and II regressing quickly, while III gives rise to the carotid arteries. Arches IV and VI form the aortic arch and pulmonary trunk. There is also a connection between pulmonary arch VI and the aortic arch called the ductus arteriosus, whose role is to bypass the pulmonary circulation before birth.

Changes in circulation at birth

At the first breath, the lungs expand, which results in an increase in pulmonary return and left atrial pressure. The tunica media muscles in the umbilical arteries contract, stopping the blood flow out of the baby. The umbilical vein then closes slowly, decreasing the blood inflow and right atrial pressure. A decrease in right atrial pressure and an increase in left atrial pressure cause the interatrial shunt to close and the foramen ovale to seal. The shunt between the pulmonary and aortic circulation, the ductus arteriosus, then also closes. The ductus venosus regresses, leaving a portal vein entering the liver and the inferior vena cava draining blood from the body to the heart.

Clinical corner

Cardiovascular anomalies are the most common life-threatening congenital defects, accounting for approximately 20% of all congenital defects in live births. Atrial septal defects are often caused by excessive cell death in the septum primum or when the septum secundum does not proliferate far enough caudally. This leaves the foramen ovale open. Ventricular defects are usually found in the interventricular septum. The most common is in the membranous part of the septum, at the site of the fusion of the conotruncal septum and the endocardial cushions. The most critical of these involves either a failure of the ventricles to seal, or the mis-alignment leading to subsequent regression of the pulmonary and aortic trunks with the right and left ventricles. Cardiovascular defects are often associated with neural crest defects. Abnormal blood flow can also lead to problems of septation of outflow tract, for example, tetralogy of Fallot, where the underlying cause is an unequal partition of the outflow tracts which leads to a pathogenetic cascade causing: (1) pulmonary stenosis, (2) interventricular septal defects, (3) overriding aorta and (4) hypertrophy of the right ventricle.

Gastrointestinal system

During weeks three and four, the embryo's flat endoderm sheet folds ventrally to form the endodermal gut tube that runs down the length of the embryo (Fig. 13.4a) [8]. The rostral part of the gut tube is incorporated into the head folds to form the foregut, while the rostral portion of the gut tube forms the hindgut. In the middle region, the midgut forms and runs continuous with the remaining yolk sac. However, further folding narrows the opening of the yolk sac until it becomes the vitelline duct, which becomes incorporated into the umbilical cord. Each region of the gut tube is determined by specific gene expression and has a specific blood supply; the foregut is supplied by the coeliac artery, the midgut by the superior mesenteric artery and the hindgut by the inferior mesenteric artery.

Also associated with the gut tube is the lateral plate splanchnic mesoderm which later differentiates into gut-associated muscular walls, connective tissue, lamina propria, submucosa and vascular elements. In early development, however, lateral plate mesoderm also differentiates into the dorsal mesentery which suspends the gut tube in the coelomic cavity (and later becomes the peritoneal cavity). A ventral mesentery also develops, but degenerates in all regions apart from the stomach and developing liver.

As the gastrointestinal tract matures in weeks 6 to 7, the endodermal epithelium proliferates into many layers. This causes many regions of the tract to lose their lumens temporarily. Recanalization however reopens these lumens by weeks 8 to 10. Mesoderm also proliferates. In the developing small intestine this mesoderm causes the epithelium to form villi and tubular glands, important for secretion and absorption. Peristaltic movement of the smooth muscle of the gut begins by week 10 following autonomic innervation.

Lung development

The lungs are composed of both endodermal and mesodermal tissue. On day 22, endodermal tissue from the lateral foregut wall forms a pouch growing out into the surrounding splanchnopleuric mesoderm. This bud, called the respiratory diverticulum, grows ventrocaudally and soon separates into right and left primary bronchials (Fig. 13.4b). The splanchnopleuric mesoderm is an important signalling centre for lung formation and later gives rise to the vascular tissue, connective tissue, cartilage and muscle within the lungs. The right and left bronchials both undergo further bifurcation in the fifth week, producing secondary bronchial buds. This budding and dividing process, known as branching morphogenesis, continues and by week 28, terminal bronchioles are formed in the sixteenth round of divisions

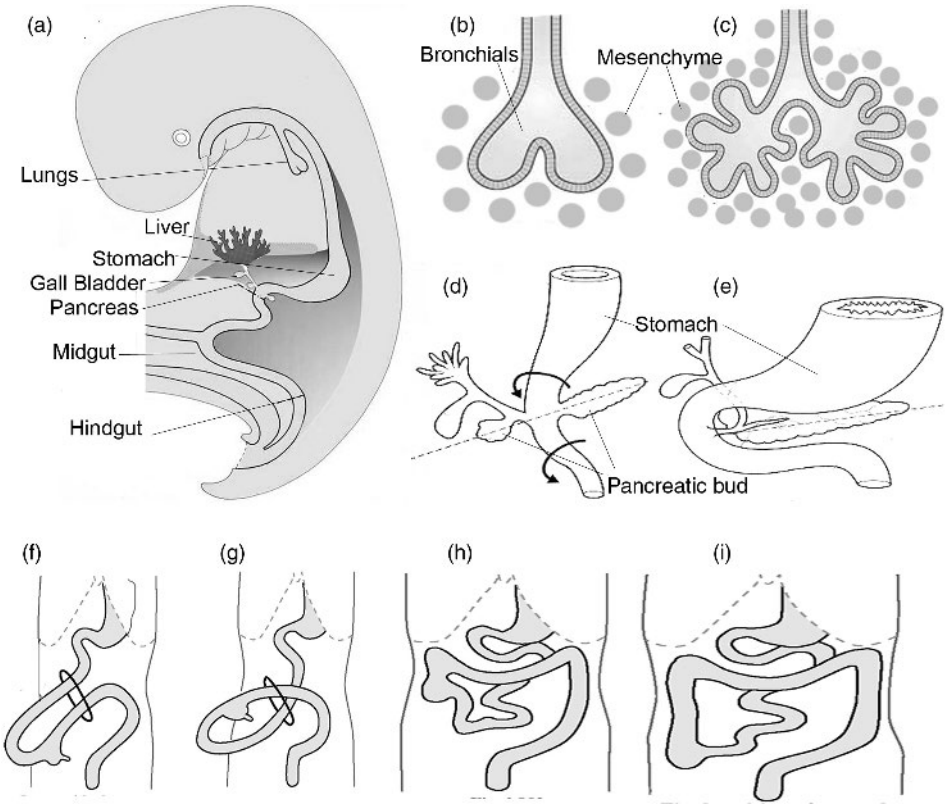

Figure 13.4 Gastrointestinal system. (a) Cross section of an embryo showing the formation of the primitive gut tube. (b) The respiratory diverticulum buds into the mesenchyme and splits into right and left bronchials. (c) These bronchials then grow by branching morphogenesis into the surrounding mesenchyme. (d) The stomach rotates and also causes the ventral pancreatic bud to unite with the dorsal pancreatic bud (e). The midgut loops at (f) week 7, (g) week 8, (h) early third month and (i) late third month where the caecum descends.

(Fig. 13.4c). The terminal bronchioles further divide and become surrounded by a capillary network. The terminal bronchioles are called terminal sacs or primitive alveoli which now mature. Further divisions of this bronchial tree are not completed until birth and additional alveoli continue to form. However, full lung maturation is not achieved until about 7–10 years of age.

Foregut development

The mouth- and tongue-most rostral region of the foregut are formed from the pharyngeal arches. Moving caudally we get the oesophagus which is initially connected to dorsal mesentery and later disappears as the oesophagus lengthens due to growth of the thoracic organs.

The stomach is the next rudiment of the foregut to form. It first appears as a dilated fusiform tube attached to the dorsal and ventral mesenteries. Due to differential rates of growth, greater in the dorsal than ventral, during weeks 5 to 6 the stomach rotates longitudinally clockwise 90° around the rostrocaudal axis (Fig. 13.4d, e). This also causes the most rostral end of the stomach to move to the left and the caudal end to the right. The dorsal mesentery, now called the dorsal mesogastrium, is also pulled to the left side. Developing within this mesogastrium is the spleen, of mesoderm origin, which is carried to the left as well. After rotation, the stomach assumes its final position with its long axis almost transverse within the abdominal cavity. The duodenum, which straddles both the caudal foregut and rostral midgut, first develops just caudal to the stomach in a C-shaped loop that projects ventrally. However, as the stomach rotates, the duodenal loop rotates to the right and comes to lie outside the peritoneum.

Clinical corner

The duodenum undergoes a process of recanalization. If this process fails, a stenosis of the duodenum results

which causes intestinal obstruction and projectile vomiting at birth [9].

The liver, gallbladder and biliary duct system develop from a bud, the hepatic diverticulum. It grows out of the epithelia of the caudal foregut early in the fourth week of development. The hepatic diverticulum extends into the mesenchyme that gives rise to the connective tissue, smooth muscle and blood vessels associated with these organs. The hepatic diverticulum divides into two parts. The larger rostral part becomes the primordium of the liver, while the other part forms the cystic diverticulum that generates the gallbladder and cystic duct. The hepatic diverticulum of the liver then branches repeatedly to form liver cords. These liver cords then disconnect to form hepatocytes which group together to form hepatic lobules which become closely associated with the mesenchyme-derived blood vessels. Hepatocytes only however begin to perform their full function after birth.

The pancreas is another organ that develops from this area, at the site of this midgut/foregut junction. It first arises at week four as two buds (the dorsal and ventral bud) emanating from the epithelial lining of the gut and growing into the surrounding mesenchyme [10]. The epithelium then undergoes a series of branching events, forming lumens that will drain exocrine products into the duodenum. The mesenchyme also plays an important role in providing cues for generating exocrine cells that produce digestive juices and endocrine cells in the Islets of Langerhans that produce insulin, glucagon and somatostatin. As the stomach and duodenum rotate, the ventral pancreatic bud is moved dorsally and comes to lie inferior to the dorsal pancreatic bud before they fuse. The ducts also fuse, allowing the ventral duct to become the main pancreatic duct feeding into the duodenum.

Clinical corner

An annular pancreas can form when the pancreas encircles the duodenum due to abnormal or reverse rotation by the ventral pancreatic bud. This may cause duodenal stenosis.

Midgut development

Derivatives of the midgut include the distal duodenum, the jejunum and ileum, the caecum and appendix, the ascending colon and proximal part of the transverse colon. As the yolk sac opening reduces to become the vitellointerstinal duct, the midgut elongates rapidly. This rapid elongation, accompanied by the enlargement of the liver and kidneys, forces the midgut intestinal loop to herniate out of the abdominal cavity into the umbilical cord at the end of the sixth week (Fig. 13.4f). However, as the gut enters the umbilical cord it rotates 90° anticlockwise. Then as the size of the abdominal cavity is increased at about week 10, the intestine re-enters the abdomen by undergoing a further 180° anticlockwise rotation (Fig. 13.4g–i). With these rotations the caecum and appendix, which were originally positioned under the liver, have moved to their adult positions in the right iliac fossa (Fig. 13.4i). The dorsal mesenteries of the colon then shortens pulling these segments up.

Clinical corner

Malrotation of the gut tube can cause twisting, which can affect the blood supply, leading to necrosis of a portion of the intestine. The intestine can also fail to re-enter the abdominal cavity. Conditions such as gastroschisis occur as a consequence of the ventral abdominal wall failing to close.

Hindgut development

The hindgut covers the dorsal one-third of the transverse colon to the upper half of the anal canal. It also includes the epithelial region of the urinary bladder and most of the urethra. The primitive gut forms an expansion called the cloaca. A band of mesenchymal tissue then divides the cloaca into the urogenital sinus (which forms the urogenital structures) ventrally and the anorectal canal dorsally. The most distal lower third region of this anorectal canal forms from an ectodermal invagination called the anal pit. Between the sixth and seventh weeks, proliferation of the ectoderm temporarily occludes the lumen of the anal canal, but this is gradually recanalized by the ninth week. The hindgut also undergoes functional maturation. The mesenchymal cells associated with the hindgut develop into muscle layers, connective tissue and blood vessels. The mesenchyme also pushes the epithelia of the recanalized epithelial layer into villi.

Clinical corner

Rectal atresia is a congenital abnormality whereby the anus and the rectum remain separated from each other. It can be caused by defects in the urogenital septum or failure of the anal canal to be recanalized.

Urogenital development

Embryologically, the urinary and genital systems are closely associated as they both arise from the intermediate mesoderm. The intermediate mesoderm is displaced ventrally following folding and forms the urogenital ridge which bulges into the coelomic cavity. Initially the urogenital system has an excretory role and enters a single common cloaca. However, the genital system loses this excretory role and develops into a separate system for reproduction.

Development of the urinary system

The urogenital system develops in a rostrocaudal progression. Beginning at the most rostral end, the pronephros develops. This is then followed by mesonephros development and then finally the metanephros develops most caudally (Fig. 13.5a) [11].

The pronephros is a temporary rudiment that first appears in the fourth week but soon degenerates. The mesonephros develops late in the fourth week and consists of functional glomeruli and mesonephric tubules which open into the cloaca. These then also degenerate, but the mesonephric ducts persist to become the efferent ductules of the testes. By the fifth week, a pair of ureteric buds sprout out of the distal part of the mesonephric duct to make the metanephric urinary system, which makes up the permanent kidney.

The ureteric buds grow into a cluster of mesenchymal cells called the metanephric blastema. This induces the ureteric bud to branch repeatedly to form collecting ducts, which drain into the renal pelvis and adult ureter. Each division of the ureteric bud is surrounded by the metanephric blastema. Cells in the blastema develop into renal tubules and nephrons. The most proximal end of these tubules is invaginated by glomerular capillaries. The mesonephric duct distal to the ureteric bud is incorporated into the wall of the developing bladder.

The kidneys also undergo positional changes during development. They first lie close to each other in the pelvis. As the abdomen grows, however, the kidneys ascend to their adult position. At week 10 the

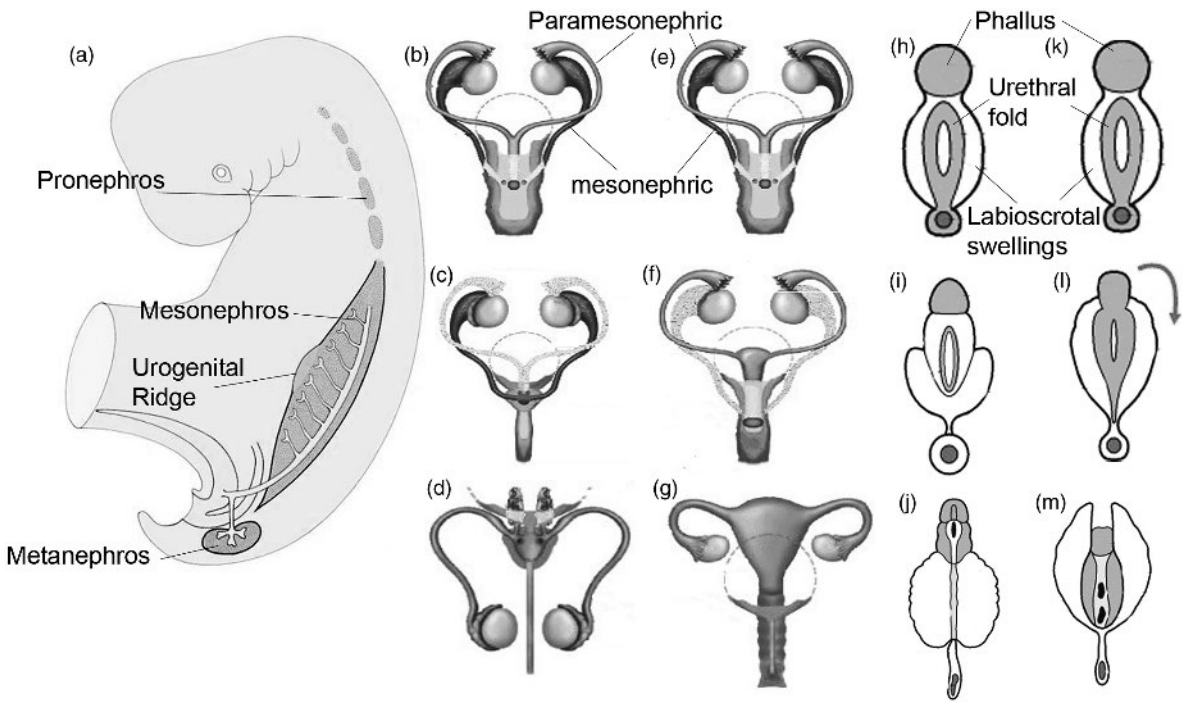

Figure 13.5 Urogenital system. (a) A cross section of a 4-week-old embryo depicting the three sets of nephric structures the pronephros, mesonephros and metanephros as well as the urogenital ridge. (b) At week 5 the indifferent stage, paired paramesonephric ducts from the urogenital ridge grow alongside the mesonephric duct. (c) In the male embryo the paramesonephric ducts degenerate leaving the mesonephric duct. (d) The testes then descend. (e) The indifferent stage. (f) In the female embryo the mesonephric duct degenerates. (g) The ovaries descend into the correct position. (h) The indifferent stage in the external genitalia at approximately 4 weeks. (i) The phallus begins to grow and lengthen in the male embryo. (j) Fusion of the urethral fold and scrotal swellings. (k) Indifferent stage. (l) The genital tubercles bends inferiorly to form the clitoris in females. (m) The urogenital folds remain separated to form the labia minora, while the labioscrotal folds form the labia majora.

metanephros kidney is fully functional, although the placenta removes fetal waste.

Development of the reproductive system

The first stage of development of the genital system, up to week 7, is called the indifferent stage, as the male and female reproductive systems cannot be distinguished from each other.

The medial portion of each urogenital ridge proliferates to form a distinct genital ridge (Fig. 13.5a). This ridge, which protrudes into the coelomic cavity, consists of the mesenchyme as well as surface ectoderm. This surface ectoderm then proliferates to make primitive sex cords that penetrate the associated mesenchyme between weeks 5 and 6.

Germ cells then migrate from the yolk sac into the sex cords by the sixth week. At this stage the sex cords start to differentiate into male and female.

The Y-chromosome associated *SRY* gene induces the TDF (testis determining factor) protein to promote the development of the testes [12]. See also Chapter 2 herein. The sex cords in the medulla proliferate to form the testicular cords, while the outer cortical cords regress and are replaced by thicker outer cells that form a capsule around the testes. Some of these testicular cords go on to form Sertoli cells that become the support cells for sperm development, while others are incorporated into the seminiferous tubules within the testis. The seminiferous tubules are separated by mesenchyme that forms Leydig cells which secrete androgenic hormones essential for the next phase of development.

In embryos where there is no Y-chromosome (i.e. female embryo) the medullary sex cords degenerate and are replaced with vascular and connective tissue. The cortical sex cord gives rise to a second generation of cortical cords that surround the oogonia and differentiate into follicle cells. Together they form the primordial follicles [13].

Sex-specific differentiation

Initially two pairs of ducts are present in both male and female embryos: (1) the mesonephric duct which served as the collecting duct for the mesonephric kidney, (2) the paramesonephric duct which runs parallel to the mesonephric duct and is formed by an invagination of the epithelium of the urogenital ridge (Fig. 13.5b and e).

In the male, the secretion of testosterone by the Leydig cells stimulates the mesonephric duct to differentiate and will later form the ductus deferens, epididymis and the opening of the urogenital sinus of the male. The paramesonephric tube regresses in response to anti-Müllerian hormone, secreted by the Sertoli cells (Fig. 13.5c). In females, in the absence of testosterone and anti-Müllerian hormone, the mesonephric duct regresses and the paramesonephric duct develops (Fig. 13.5e). These paramesonephric ducts develop into oviducts, the uterus and upper third of the vagina [13]. The lower part of the vagina is formed from the urogenital sinus endoderm.

Sexual differentiation of the external genitalia begins during the ninth week and is completed by week 12 [14]. As with the internal sex organs, the external organs start off sexually indifferent (Fig. 13.5h and k). The proliferating mesenchyme first forms swellings, cloacal folds, around the cloacal membrane at week 3. The cloacal folds then unite ventrally to form the genital tubercle. Labioscrotal swellings and urogenital folds soon develop on each side of the cloacal membrane which form the scrotum in males and the labia majora in females (Fig. 13.5j and l). The genital swellings fuse in males only. The genital tubercle then elongates to form the primordial phallus. In males, hormonal influences further elongate the genital tubercle to form the penis and urethral folds fuse to form the penile urethra (Fig. 13.5j). In females, the genital tubercle forms the clitoris and the urethral folds remain separate to form the labia minora (Fig. 13.5m). During week 6, as the cloaca divides internally into the urogenital sinus and anorectal canal, the external cloacal folds also become divided into urogenital folds and anal folds. The cloacal membrane also divides into the urogenital and anal membranes, which break down, creating openings. In both sexes the gonads descend. The testes descend into the scrotum (Fig. 13.5d), and the ovaries descend a shorter distance caudally within the pelvic cavity (Fig. 13.5g).

Clinical corner

Sexual differentiation defects can occur, for example, in females when there is an absence of one X-chromosome, called Turner's syndrome, leading to ovary degeneration, while in males an extra X-chromosome, called Klinefelter's syndrome, causes small testes and a lack of, or low sperm count. Abnormalities also include pseudohermaphrodic individuals, whereby the internal organs do not match the external genitalia. For example, androgen insensitivity syndrome, where the individual is XY genetically and has female external organs.

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Chapter 14

Global perspectives in reproductive health and fertility

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Introduction

> *Reproductive health is a state of complete physical, mental and social well-being, in all matters relating to the reproductive system and its functions and processes, not merely the absence of disease or infirmity.*
>
> 4th International Conference on Population and Development, Cairo, 1994 [1]

Reproductive health could be defined simply as the absence of disease or dysfunction of the reproductive organs. However, the definition should encompass much more than biological systems. Reproductive health includes the capacity to control one's own fertility and to have a safe pregnancy, as well as healthy children. In addition, one should be able to have sex without risk of infection and within relationships which promote equality, choice and respect.

The definition concerns individuals, partnerships and family life. Reproductive health is central to one's happiness and well-being, and should be considered a basic human right.

Why is reproductive health important?

Poor reproductive health is a major contributing factor to morbidity and mortality worldwide. In fact, unsafe sex is the second most important risk factor leading to disability or death in developing countries. Every year, approximately 340 000 women die and 210 million have life-threatening complications in pregnancy, there are 340 million new cases of the four most common STIs and 5 million new HIV infections [2, 3].

Reproductive health can be influenced by individual, local, societal and political factors, as shown in Figure 14.1.

In theory, in developed countries, we are well equipped with the necessary tools to provide good reproductive health: safe and effective contraception, treatment for common STIs, evidence-based interventions for prevention and prompt treatment of maternal complications, etc. Where these services are adequate and accessible, good reproductive health is achieved. However, there are still huge inequalities and barriers standing in the way of good reproductive health for all globally. The consequences of poor reproductive health are far-reaching, and thus the topic should be a priority at local, national and international levels.

This chapter will attempt to discuss the global perspective on reproductive health and fertility by focusing on four main reproductive rights.

Every woman has the right to

- a safe pregnancy
- plan her family
- a family
- have safe sex

Every woman has the right to . . . a safe pregnancy

From newly published WHO data, it is estimated that approximately 340 000 women now die as a result of pregnancy or childbirth-related complications each year, despite the availability of effective and affordable interventions to prevent these tragedies [2]. Ninety-nine per cent of the deaths occur in developing countries, the majority in Sub-Saharan Africa and South Asia, as shown in Figure 14.2 [4].

Thanks to international efforts, as part of the Millennium Development Goals (MDGs), the maternal mortality figures have fallen dramatically from

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

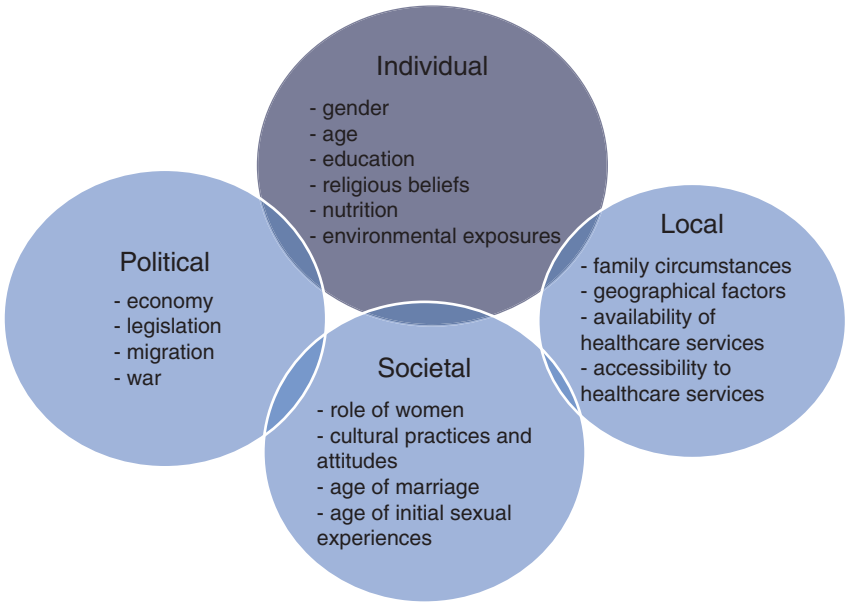

Figure 14.1 Illustration of the numerous factors contributing to reproductive health.

more than half a million deaths in 2005, although not in all countries due in part to the HIV epidemic, which is adding to the burden of pregnancy in these resource-poor settings.

The tragic inequalities in maternal health are illustrated by the high maternal mortality ratio (MMR) in developing countries: for example, the MMR in Malawi is over 1000 maternal deaths per 100 000 live births, compared to a figure of 4 in Italy [2].

Maternal health is clearly important for the individual woman, as well as her baby, in the preconceptual, pregnancy and postpartum periods. It also almost certainly influences child and adult health in the long term, which in turn affects maternal health in the next generation. Hence, improving maternal health has major potential advantages for society as a whole.

> *A mother's unnecessary death in childbirth isn't just a human tragedy. It's also an economic and social catastrophe that deprives her surviving children of nurture and nutrition, and too often of the chance of education.*
>
> Julian Schweitzer, Acting Vice President of Human Development Department, World Bank [5]

Why do women die?

The causes of maternal deaths globally are well described, with hemorrhage accounting for the majority (25%), followed by infections (15%) and unsafe abortion (13%) [6].

Although these are the leading causes of maternal mortality worldwide, they are much more prevalent in developing countries. For example, postpartum hemorrhage (PPH) causes a third of maternal deaths in Africa, compared to only 13% in developed countries, where safe, effective and inexpensive treatments such as oxytocin are more readily available [7].

To prevent and treat such maternal complications, several actions are required. First, potential complications should be recognized in the antenatal period where possible, which requires provision of good-quality antenatal care. WHO recommends at least four visits during pregnancy; however, at present, one-third of women receive no antenatal care at all in developing countries [8].

Of course, not all complications, e.g. obstructed labour and PPH, can be reliably predicted antenatally. Therefore, a 'trained birth attendant' (TBA) – midwife, doctor or skilled birth attendant – should be present at the birth to recognize and manage potential complications, and refer the patient to a higher level facility where appropriate. However, only 63% of births globally are attended by trained staff: ranging from 99% in developed, to 34% in less developed, countries [9]. When births are not attended by a TBA, complications may go unrecognized with potentially fatal consequences. Even if these are recognized, delays in transferring women to appropriate health facilities, possibly as a result of poor infrastructure or transport, can be a problem in developing countries.

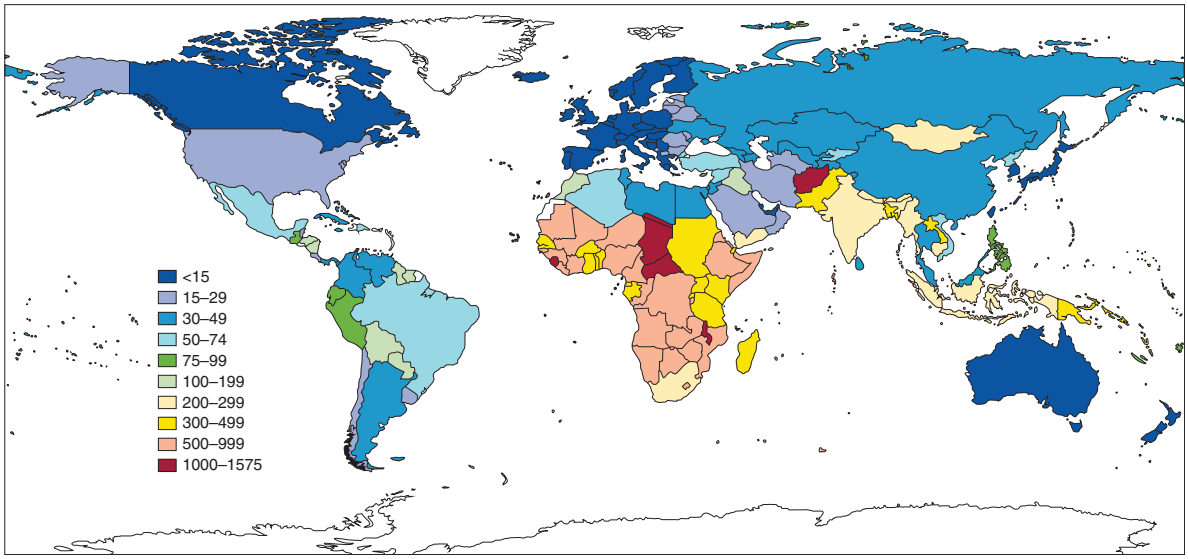

Figure 14.2 Maternal deaths per 100 000 live births in 2008. Source: M. C. Hogan, *et al.*, Maternal mortality for 181 countries, 1980–2008: a systematic analysis of progress towards Millennium Development Goal 5. *Lancet* **375**, no. 9726 (2010): 1609–23.

Once at an appropriate centre, further delays may occur due to shortage of staff and/or a lack of resources. Even when suitable healthcare is available, there are numerous other barriers preventing access, as shown in Figure 14.3.

What is the solution?

The causes of maternal deaths are well known. How to prevent and manage these problems effectively is also well established. Therefore, what is important is to ensure there is adequate provision and utilization of the appropriate services for pregnant women. This will require change at many different levels. For example, there is a need to improve education and empower women to have the courage to access maternal services; provide TBA training programmes for faster recognition and referral of labour complications; and invest in better equipped health facilities.

Innovative solutions exist in the form of the FIGO Safe Motherhood and Newborn Health Projects, such as a financial incentives scheme in India, which led to a tenfold increase in the number of mothers delivering at formal health facilities [10]. However, the real challenge lies in implementing such schemes on a global scale, as they must be adequate and appropriate for individual regions/countries with different needs, as well as different social, cultural, political and economic circumstances. The overall goal is to provide adequate obstetric care for everyone, which will confine preventable maternal deaths to history.

Every woman has the right to . . . plan her family

The 4th ICPD, 1994, specifically states:

> *Reproductive rights include the basic right of all couples to decide freely and responsibly the number, spacing, and timing of their children, and to have the information and means to do so.* [1]

To exercise this right, a woman needs access to safe and effective contraception, but there are striking global disparities regarding contraceptive use, particularly between developed and developing countries. For example, in some parts of Africa, less than 10% of married women use any form of contraception, which is in stark contrast to Northern Europe, where more than 80% of married women are doing so. Not surprisingly, this results in significant differences in birthrates: 1.4 children/woman on average in Europe, compared to 5.1 in Africa, and as high as 8.0 in Niger. Furthermore, within countries with high birthrates, the highest figures tend to be in the poorest proportion of the population [11].

Why is the use of modern contraceptives so low in these countries? It could be explained by women choosing not to use contraception because of opposing

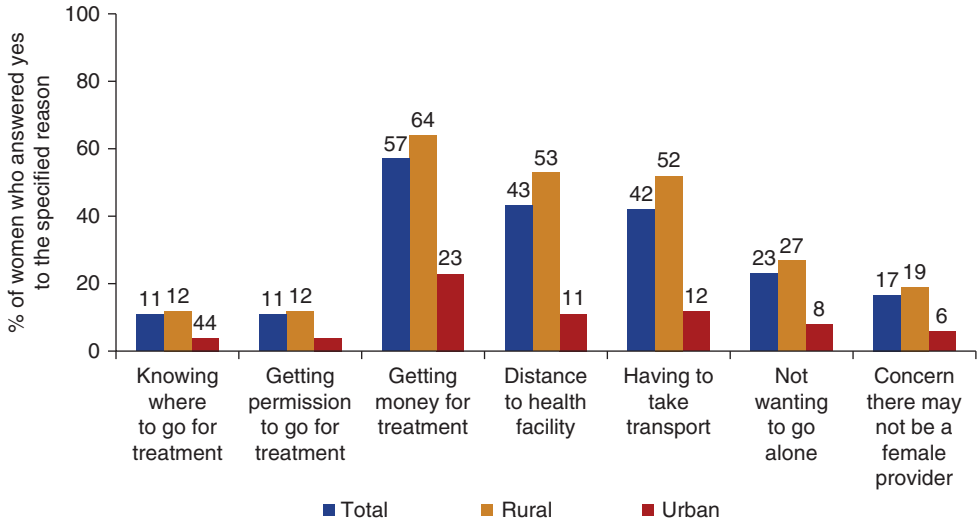

Figure 14.3 Surveys carried out in 24 African countries reveal some of the main problems preventing women in Africa accessing health care during pregnancy. Source: Macro International Inc., 2008. MEASURE DHS STAT compiler. http://www.measuredhs.com, July 16 2008. Secondary analysis conducted by the WHO Department of Making Pregnancy Safer. Taken from: Islam *et al.*, Women still deprived of access to lifesaving essential and emergency obstetric care. *Intl J Gyn Obstet* **106** (2009): 120–4.

religious and/or cultural beliefs. However, there are data showing that a high proportion of women in the developing world have an *unmet need* for contraception, as many as 1 in 4 in Sub-Saharan Africa. That is to say they wish to use contraception, but cannot access it [4].

Why is family planning so important?

At an individual level, as described above, a woman should have the right to plan her family. Removing that right affects her general health because of problems related to the number and spacing of pregnancies, and the age of childbearing. For example, a woman who conceives at a young age is at increased risk of premature labour and of dying, which in turn affects her child's health if it survives. It is estimated that meeting the world's need for contraception could prevent 32% of maternal and 10% of infant deaths [12]. Fertility rates also affect family circumstances, and may exacerbate poverty and poor education. On a larger scale, countries with high fertility and fast population growth have increased demand on natural and economic resources, perpetuating national poverty and socioeconomic problems. Another major advantage of improving access to family planning would be a reduction in the number of unsafe abortions.

The issue of unsafe abortion

Unsafe abortion is defined as 'a procedure for terminating an unintended pregnancy by individuals without the necessary skills, or in an environment that does not conform to minimal medical standards, or both' [13]. It is estimated (see Fig. 14.4) that approximately 19 million unsafe abortions take place every year, 97% of which occur in developing countries [14].

The result is approximately 68 000 deaths per year, which is 13% of global maternal mortality. Many more women suffer serious morbidity: approximately 20% of unsafe abortions result in a reproductive tract infection, leading to chronic infection and infertility rates of 5% and 2% respectively. It may also lead to problems in future pregnancies, such as spontaneous miscarriage, ectopic pregnancy and premature delivery. In addition, women who suffer such reproductive disabilities are often maltreated and discriminated against within their family and communities [13].

Why is unsafe abortion so prevalent?

High rates of unsafe abortion are associated with a high unmet need for contraception [13]. There also appears to be a strong relationship between the legal status of abortion within a country and the mortality rate from unsafe abortion. For example, in Romania, in the 1980s, when

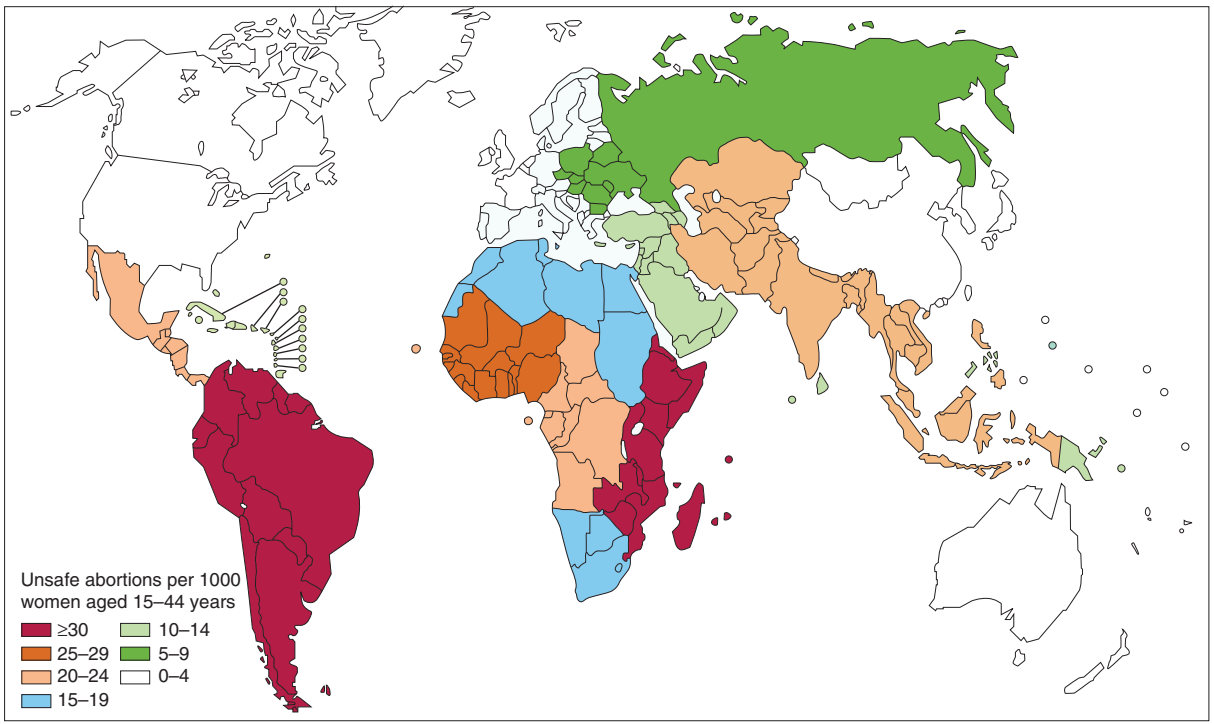

Figure 14.4 The estimated yearly incidence of unsafe abortion per 1000 women aged 15–44 years, 2000. Source: WHO. *Unsafe abortion. Global and regional estimates of the incidence of unsafe abortion and associated mortality in 2000.* 4th edn. Geneva: World Health Organization, 2004. Taken from Glasier *et al.*, Sexual and reproductive health: a matter of life and death. *Lancet* **368**, no. 9547 (2006): 1595–607.

legislation was highly restrictive, mortality from unsafe abortion was approximately 148 per 100 000 live births. In 1989, the law changed under a new political regime and abortion was made more available, with fewer eligibility criteria. As a result, the mortality rate from unsafe abortion fell dramatically to 9 per 100 000 live births [15].

It follows, therefore, that if countries made legislation on abortion less restrictive, it is highly likely that maternal morbidity and mortality rates from unsafe abortion would fall. However, as many cultures and religions are strongly opposed to abortion, it is perhaps unrealistic to imagine that such societies will ever legalize abortion and/or provide better access to modern contraceptive methods.

Why is there an unmet need for contraception?

Four key barriers have been identified in preventing women from accessing contraception worldwide [12]:

- Insufficient knowledge about contraceptive methods and how to use them
  - where education about reproductive health is poor
- Fear of social disapproval
  - some cultures and religions oppose the use of contraceptives
- Fear of side effects and health concerns
  - related to insufficient knowledge of the methods and how they work
- Woman's perception of husband's opposition
  - some cultures consider a woman's role is to bear children and her 'success' as a wife may be judged on the basis of her fertility

Unmet need for contraception is also associated with poverty. For example, in Asia and Latin America, unmet need in the poorest 20% of the population is double that in the wealthiest 20%. This may be because wealth influences the four key barriers, through educational achievement and social background [12].

What's the solution?

Some argue that improvement in living standards, better education and social empowerment of women are the ways to improve access to family planning in developing countries. This makes sense, as the evidence clearly points to major socioeconomic factors contributing to unmet need. However, tackling all these issues is a huge task, and change may not occur for years or even decades. In the meantime, there have been numerous initiatives to provide family planning services to those in need.

Local family planning programmes can be extremely beneficial but, importantly, they must fit their social context. They need to be targeted to suit the needs of the intended audience. Programmes should attempt to create an environment in which using contraception to have smaller families becomes the norm. This may require liaison with religious or traditional leaders, local community workers and the media.

Contraceptive methods need to be accessible. At the most local level, community-based clinics are necessary, particularly in rural areas. These have the advantage of being run by local people who share the same language and customs, which may help to make contraceptive use more acceptable. People should also be able to access contraceptives from commercial outlets as well as larger health facilities, although women still need to be provided with education, as lack of knowledge about the methods available is a barrier to use.

Key features of effective family planning programmes

1. Should promote small family size and increase awareness and acceptability of contraception, with initiatives appropriate to that society
2. Make accessing contraceptives easy and approachable
3. Make acquiring contraception affordable
4. Promote the most effective methods

The cost of buying contraception needs to be considered because this is a major barrier to many women, especially as the highest unmet need occurs in the poorest populations. Ideally, cost should not be a deterrant. Most governments do provide free/low-cost contraception, but the sustainability of such a strategy is unclear in countries with current low levels of contraceptive use if they suddenly intiated family planning programmes and then experienced a surge in use, which drained all available funding. Despite these concerns, it still appears a highly cost-effective intervention to provide many other health benefits such as reduced maternal mortality and morbidity, reduced family poverty with lower fertility rates, and so on.

The best available data show a skew towards one or two favoured methods in countries: for example, 61% of contraceptive users in Egypt use the intra-uterine device, whereas in Bangladesh, almost half the population favour the oral contraceptive pill [12]. Although certain methods are more effective than others, and family planning programmes should be encouraged to

promote the most effective ones, it must always be appreciated that some methods will be more acceptable to certain societies/cultures.

Clearly, family planning should become a higher priority, considering the enormous unmet need, particularly in the poorest populations: not only because it is a basic human right for a woman to be able to 'plan her family', but also because of the more general benefits to society as a whole.

Every woman has the right to . . . a family

As argued above, every woman should have the right to control her fertility. However, 'family planning must also mean planning for families', according to Mahmoud Fathalla, former president of the Federation for International Gynecology and Obstetrics (FIGO). This was highlighted in the ICPD, Cairo 1994 conference, which concluded that 'prevention and appropriate treatment of infertility, where feasible' was important [1].

The burden of infertility

Infertility is thought to affect approximately 15% couples of reproductive age worldwide [16]. Its consequences can be far-reaching, not only causing significant psychological distress, but also having social and economic effects. In certain cultural contexts, women may be much more vulnerable to the negative consequences of infertility (even in the presence of male factor infertility as the primary problem). For example, a study of infertile women in Pakistan revealed that 20% were threatened with divorce; 25% were told they may be returned to their parents' home; 33% of the husbands threatened to re-marry; and 10% of the women suffered verbal and physical abuse from the husband and his family [17].

The problem of infertility in resource-poor countries

Although it is a worldwide problem, the causes of infertility vary between developed and developing countries. For example, in Sub-Saharan Africa, tubal factor infertility is the cause in 85% of women, compared to a global average of 33%. In developing countries, this is associated with unprotected sex at a young age (resulting in sexually transmitted infections (STIs), e.g. chlamydia and gonorrhoea), unsafe abortions, postpartum infections and fistulae from obstructed labour [18], all of which could be significantly reduced with appropriate reproductive health prevention strategies.

Sadly, infertility in resource-poor countries is a seriously under-recognized and neglected problem, because maternal health and family planning tend to dominate resource allocation. Despite this, many IVF units have opened in recent years in countries such as India and China, highlighting that there is a definite demand for infertility treatment, even in settings where fertility and maternal mortality rates are high. However, there are major barriers to the treatment of infertility in developing countries. First, population growth of any sort is often considered detrimental, putting a strain on natural and economic resources and exacerbating poverty. Even when assisted reproduction is accepted, the services can be prohibitively expensive in a funding environment that is almost exclusively private. Success rates may also be low because of poor training and a lack of regulation. Finally, in public health terms, it is justified to question the advisability of allocating funding to such a cause when resources are limited, despite the associated misery and distress at the level of the individual couple.

How to approach infertility in resource-poor countries

In terms of the diagnosis and treatment of basic infertility, the pathways need to be affordable, evidence-based and streamlined to reduce cost and increase accessibility. For example, relatively simple treatments such as clomiphene, with adequate monitoring, to induce ovulation in cases of anovulatory infertility (WHO Class II) should always be offered before considering expensive IVF.

However, there is an even stronger argument for investing in the prevention of infertility in resource-poor countries rather than treatment, which would bring multiple benefits. Prevention of some of the major causes, e.g. STIs and unsafe abortions, could also be extremely cost-effective in both economic and healthcare terms.

Every woman has the right to . . . safe sex

Unsafe sex is considered to be the second most important risk factor of disease, disability and death in developing countries, and the ninth most important in developed countries [3].

The burden

There are 340 million new cases of curable STIs (gonorrhea, chlamydia, syphilis and trichomonas) annually and

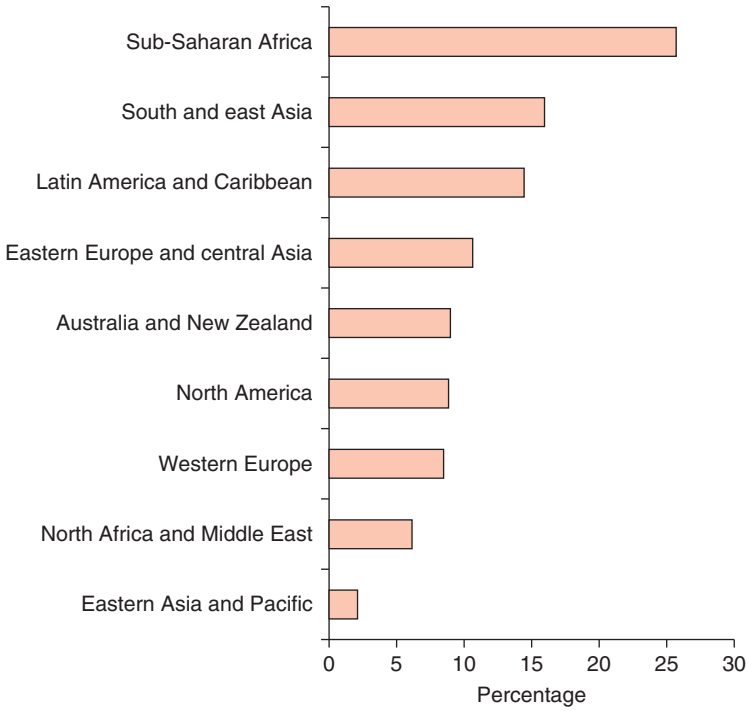

Figure 14.5 The combined frequency of infection from gonorrhea, chlamydia, syphilis and trichomonas in people aged 15–49 years. Source: WHO. Global prevalence and incidence of selected curable sexually transmitted infections: overview and estimates. Geneva: World Health Organization, 2004. Taken from Glasier *et al.*, Sexual and reproductive health: a matter of life and death. *Lancet* **368**, no. 9547 (2006): 1595–607.

18.6 million disability-adjusted life years (DALYs) are lost each year from syphilis, gonorrhea and chlamydia alone. These figures are an underestimate of the global burden of STIs, as they do not include many of the other infections such as human papilloma virus (HPV), herpes simplex virus (HSV), hepatitis B, and others [19].

STIs are much more of a burden in some geographical areas than others. For example, approximately 2% of the East Asian population, aged 15–49, are affected by an STI, compared to 25% in Sub-Saharan Africa. Developed countries tend to have a much lower rate of STIs compared to developing countries, as shown in Figure 14.5, despite similar sexual behaviour [3].

Who is affected by STIs?

The individual

It could be argued that women are more likely to acquire an STI than men. Certainly, transmission rates from men to women are higher than from women to men, due to the surface area of the genital tract epithelium. Social factors also make women more vulnerable to STIs: for example, in certain cultures it is acceptable for men to adopt high-risk behaviours such as having multiple partners. Women in poverty may be coerced into becoming sex workers, putting them at risk of infection.

Furthermore, it could be argued that the pathological consequences of an STI are greater for women than men. A case in point is HPV infection, which has a much greater impact on the health of women, causing approximately half a million cases of cervical cancer per year, with 250 000 deaths. STIs tend to manifest later in women than men, by which time irreversible damage has often occurred. For example, fallopian tube damage following an STI accounts for 30% of female infertility [20]. Women also suffer more social consequences as a result of infection, such as divorce, social exclusion and verbal and physical abuse from a partner and his family.

Lastly, it is important to note that the presence of one genital tract infection such as chlamydia can increase the risk of acquiring another, such as HIV. Likewise, HIV is associated with an increased risk of acquiring other STIs.

Partnerships

STIs affect not only the health of the infected woman and her sexual partners, but also another important partner – the fetus. It is estimated that there are 1.6 million pregnant women with undiagnosed syphilis in Sub-Saharan Africa, among whom 1 in 4 women have stillbirths and 1 in 5 babies will also die soon after birth because of syphilis. Screening pregnant women could prevent around half a million fetal deaths per year.

Table 14.1 Summary of effects of STIs, and interventions at individual, partner and population levels. Inspired by [22]

	Effects	Interventions
Individual	Mortality Reproductive health May increase risk of acquiring HIV Infertility Social consequences	Prevention, e.g. -condoms -male circumcision -vaccination Diagnosis and management, e.g. -improving rapid tests -effective single-dose therapies
Partnerships	Sexual partnerships -partner may acquire the infection Maternal partnership with fetus -stillbirths and perinatal mortality -neonatal disability, e.g. blindness	Partner notification -patient -healthcare professional
Population	Cause/exacerbate epidemics of the infection Exacerbate HIV epidemic Economic consequences as a result of DALYs lost	Screening -opportunistic -at-risk populations -antenatal Presumptive treatment programmes Vaccination programmes

Maternal gonorrhea in comparison causes blindness in an estimated 4000 neonates every year [3].

The population

Untreated STIs can perpetuate further transmission of infection and contribute to epidemics, affecting large numbers of people. Furthermore, certain infections increase the risk of acquiring HIV, exacerbating the HIV epidemic. STIs result in millions of lost DALYs every year, preventing people working and thus affecting the economy.

The solutions

Considering the individual, partner and population effects of STIs, we can think about intervention strategies in the same way (summarized in Table 14.1).

Individual

Primary prevention of STIs by practising safe sex and condom use would be an ideal, low-cost solution. However, there are considerable barriers, including poor education and a general unwillingness among many males to adopt such practices. It may be especially difficult for a woman to negotiate condom use with a partner in cultures with gender inequality. There are other interventions with proven effect in reducing the transmission of STIs, including male circumcision, which reduced HIV transmission by 60% in a recent trial [21]. Vaccines against hepatitis B in gay men, and against HPV in unexposed women, are further examples of how individuals can be protected.

Improving the diagnosis of STIs with better 'point-of-care tests', such as a recently developed dipstick with monoclonal antibodies for chlamydia antigens [22], could increase the number of infected individuals receiving effective treatments, particularly if offered in a 'one-stop shop' fashion. This would be most useful in poor rural or developing areas with limited infrastructure and/or social stigmatization associated with STIs, where repeat visits to health facilities are less feasible.

Partnerships

Partner notification is an excellent way to ensure that exposed partners are tested and treated appropriately. This should preferably be done by the individual, but it can be extremely challenging as he/she may be at risk of blame, social stigma, abuse and relationship breakdown [22].

Population

Screening is a highly effective intervention that can have population benefits in terms of STI control. Currently, there are opportunistic chlamydia screening programmes: when individuals meet with a healthcare professional, they are offered the chance to be screened if they wish. However, there are no data as to whether opportunistic screening is effective at reducing chlamydia prevalence.

Antenatal screening programmes for maternal syphilis, already in place in developed countries, should be a major priority particularly in regions with a high prevalence such as Sub-Saharan Africa,

where they could have huge benefits in terms of both maternal and fetal health.

It has been argued that other STIs are being neglected as a health priority since the emergence of HIV, and as financial support for HIV has increased, that for other STIs has decreased [22]. This is despite the fact that the data clearly show that other STIs, such as syphilis, chlamydia, gonorrhea and HPV, are creating a huge health and economic burden, and may even increase HIV transmission in some cases. There is a clear case for re-evaluation of investment in such diseases.

The importance of a global perspective for clinical embryology

Clinical embryology is the study of how to translate knowledge about the formation, growth and development of the embryo and fetus into clinical practice to achieve a healthy pregnancy. However, the healthy development of the fetus is strongly dependent on good reproductive health. Fetal health and survival will in turn affect long-term adult health, which will influence reproductive health in a never-ending cascade of events. Clinical embryology can therefore be considered part of a health cycle, at the centre of which is reproductive health.

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Chapter 15

Fertility control and contraception

Enda McVeigh

Couples or individuals seeking advice on controlling their fertility may do so for a number of reasons, which may include pregnancy avoidance, family 'spacing' or following completion of their family. When seeking advice on contraception, individuals and couples should have a confidential discussion that is non-judgemental, unbiased and respectful of the diversity of their cultural and faith traditions. In the case of patients under 16 years of age (age of consent in the UK), the Fraser Guidelines are employed as listed below (issued after the 1985 Gillick case).

To assess whether the girl under 16 years of age is 'Gillick Competent', the healthcare practitioner:

1. Must ensure the young person understands the potential risks and benefits of the treatment/advice given.
2. Is legally obliged to discuss the value of parental support, yet the client must know that confidentiality is respected whether or not this is given.
3. Should assess whether the client is likely to have sexual intercourse without contraception.
4. Should assess whether the young person's physical/mental health may suffer if not given advice on contraception or supplies.
5. Must consider if it is in the client's best interests to give contraception without parental consent.
6. Must respect the duty of confidentiality that should be given to a person under 16, and which is as great as that owed to any other person.

Attempts should first be made to involve a parent in the decision to prescribe a 'medical' method of contraception. Yet it can be good practice to prescribe, for example, the combined oral contraceptive pill ('The Pill') in the absence of such parental support.

Features of the ideal contraceptive

Like many things in medicine, the 'ideal' treatment is seldom available. Nevertheless, consideration of the factors affecting the successful use of contraception lend support to the need for as many as possible of the ten 'ideal' features, which include:

- 100% effective (with the default state as contraception)
- 100% convenient (forgettable, non-coitally related)
- 100% safe, free of adverse side effects (neither risk nor nuisance)
- 100% reversible, ideally by self
- 100% maintenance-free, meaning needing absolutely no medical or provider intervention (with potential pain or discomfort): whether initially, or during usage, or to achieve reversal
- 100% protective against sexually transmitted infections (STIs)
- Having other non-contraceptive benefits
- Cheap, easy to distribute
- Acceptable to every culture, religion and political view
- Used by or at least clearly visible to the woman, who most needs to know it has worked

It is difficult to decide priority for such features. Indeed, all the available means of contraception will have some but not all of these features. However, the first six factors are considered paramount.

Sexually transmitted infections (STIs)

The prevalence of STIs in the UK is rising, of which the most common conditions now are chlamydia, *Chlamydia trachomatis* (acquired by > 10% of sexually

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

active teenagers), non-specific urethritis, and wart virus infections. In the UK, women at 'higher risk' of infections (particularly with *Chlamydia trachomatis*) are those under the age of 25 years, alongside a partner change in the previous 12 weeks, and more than one partner in the past 12 months.

Sexual history should be seen as part of the initial consultation for all contraceptives. The sexually active of all ages should be advised about minimizing their risk of sexually transmitted infections, including the human immunodeficiency virus (HIV). It is essential to promote the condom as an addition to the selected contraceptive, whenever infection risk exists.

Relative effectiveness of available methods

Failure rates of contraceptive methods are usually expressed as 'failures per 100 woman-years'. In discussing failure rates with individuals or couples, it is important to put this in a language that they understand. Effectiveness rates vary on the ability of the user to 'utilize' the contraception correctly. Thus, there will be a 'perfect use' effectiveness, which means the method is used both consistently and correctly, whereas 'typical use' illustrates how a typical user will use the contraception (Table 15.1).

To illustrate the 'perfect user' terminology: a figure of 10 per 100 woman-years for a 'perfect user' would mean that in a population of 100 users, 10 women might be expected to conceive in the first year of use, or one woman would have an 'evens' chance of having an unplanned pregnancy after 10 years of its use.

Table 15.1 Effectiveness of different types of contraceptives: Perfect use and typical use.

Birth Control Method	Effectiveness Rate (%)	
	Typical Use 1	Perfect Use 2
Abstinence (no sexual contact)	100.00	100.00
IUD – Mirena	99.90	99.90
Male sterilization (vasectomy)	99.85	99.90
NuvaRing	92.00	99.70
Evra Patch	92.00	99.70
Oral contraceptive pill (OCP)	92.00	99.70
Depo Provera	97.00	99.70
Female sterilization (tubal ligation)	99.50	99.50
IUD – Copper T	99.20	99.40
Male condom	85.00	98.00
Natural family planning	75.00	96.25
Withdrawal	73.00	96.00
Female condom	79.00	95.00
Diaphragm	84.00	94.00
Spermicides	71.00	82.00
Chance	15.00	15.00

Eligibility criteria for contraceptives

Depending on the medical history of women, different contraceptive methods may be relatively or completely contraindicated. Like most decisions in clinical medicine, a risk-benefit analysis needs to be performed with the patient. The World Health Organization (WHO) system for classifying contraindications is described in the documents issued by the WHO. In 2006, the Faculty of Family Planning and Reproductive Healthcare (FFPRHC) issued the UK medical eligibility criteria (UKMEC), a UK-adapted version of the WHO medical eligibility criteria (WHOMEC) developed by its Clinical Effectiveness Unit. This version has some important differences and is downloadable from www.ffprhc.org.uk.

There are four categories of contraindication as indicated by the WHO:

WHO 1: A condition for which there is no restriction for the use of the contraceptive method

WHO 2: A condition where the advantages of the method generally outweigh the theoretical or proven risks

WHO 3: A condition where the theoretical or proven risks usually outweigh the advantages, with an alternative method usually preferred

WHO 4: A condition that represents an unacceptable health risk

Clinical judgement is required, always in consultation with the contraceptive user, especially: [1] in all *WHO 3* conditions; or [2] if more than one condition applies. As a working rule, two *WHO 2* conditions move the situation to *WHO 3*; and if any *WHO 3* condition applies, the addition of either a *WHO 2* or a *WHO 3* condition normally leads to the application of *WHO 4*, i.e. 'Do not use'.

Fertility awareness and methods for the natural regulation of fertility

For some couples, religious reasons may prevent them from using artificial means of contraception. For such couples, 'fertility awareness' may be the only method available. These methods are capable of being much more reliable than the old calendar rhythm method, if there is correct and consistent use. However they still remain 'very unforgiving of imperfect use'. To encourage 'perfect' use, an understanding of the background physiology is important. Key facts that should be explained to the couple include:

1. An average fertile man's ejaculate contains around 3–400 million sperm.
2. The acidic vagina environment can kill sperm in a matter of hours. However, in estrogen-primed cervical mucus and upper genital tract fluid, average sperm survival is around 3 days.
3. In rare individuals, or rare cycles in which favourable mucus appears early, fertilization can be as long as 7 days after ejaculation.
4. The average fertilizable life span of the egg(s) after ovulation is ~17 hours, with a range up to a maximum of 24 hours.
5. Adding the lifetime of the sperm to that of the egg gives a 'fertile window' of 7–8 days, whose length, while constant, shows intra- as well as inter-individual variation.

Maximum reliability will require many days of abstinence, especially early in the cycle. For maximum efficacy with any of the methods, unprotected intercourse should preferably, following good evidence of ovulation, be confined to the days after the ovum is no longer fertilizable.

Markers of ovulation

To use the 'fertility awareness' means of contraception, it is crucial that the user can identify, with some degree of reliability, subjective signs of ovulation. These include a sustained rise in basal temperature, 72 hours at least 0.2°C above the preceding six days' values, and observations of mucus at the vulva. The ovulatory mucus becomes increasingly fluid, glossy, transparent, slippery, and stretchy, resembling raw egg white, under the influence of follicular estrogen. The peak mucus day can be recognized retrospectively as the last day with such features, before an abrupt change to a thick and waxy appearance (under the influence of progesterone).

The postovulatory infertile phase is defined as beginning on the evening of the fourth day following the peak mucus day, provided this is also after the third of the higher morning temperature readings. Relying on both the above signals for the onset of the post-ovulatory infertile phase, and using that alone for unprotected intercourse, yields very acceptable failure rates of 1–3 per 100 woman-years. The preovulatory infertile phase is much more difficult to accurately identify. Indicators include the first sign of any mucus at all, detected by either sensation or appearance, and a calendar calculation of the shortest cycle minus 20 (or better, 21) to give the last 'infertile' day where at least six cycle lengths are known. This can be enhanced by the 'Doering rule', in which 7 days are subtracted from the earliest cycle day of documented temperature shift. Whichever of these two indicators comes first indicates the requirement to abstain.

Relying on both phases is only to be recommended to those who can accept a pregnancy, since calculations and mucus observations do not reliably predict ovulation far enough ahead to eliminate (over many months or years) the capricious survival of that last-surviving sperm which could cause conception. Following birth (up to approximately 6 months) and in the 5–10 years leading up to the menopause, temperature and mucus estimations are unreliable and/or give numerous 'false alarms'. This is due to the fact that some cycles are anovulatory but with sufficient estrogen to produce slippery mucus.

Such information also aids in utilizing the lactational amenorrhea method (LAM). This relies on the fact that ovulation is delayed among women who fully or nearly fully breastfeed their babies. It usually takes between one and three months for a woman to begin to ovulate, and for her cycle to return to normal after stopping breastfeeding.

Advantages of methods based on fertility awareness

Although the fertility awareness method of contraception is not ideal, it does have some advantages, including:

- Being completely free from any known physical side effects for the user.
- Being acceptable to many religious and cultural views, not only those of Roman Catholics.

- Methods are under the couple's personal control.
- Methods readily lend themselves, if the couple's scruples permit, to the additional use of an artificial method such as a barrier at the potentially fertile times, including during the less-safe first 'infertile' phase.
- Once established as efficient users, after sufficient teaching, no further expensive follow-up of the couple is necessary.
- Understanding of the methods can also help couples who then wish to conceive.

'PERSONAR' – the Unipath personal contraceptive system

This innovative product, first marketed in 1996, consists of a series of disposable test sticks and a hand-held, computerized monitor. Test sticks are dipped in the user's early morning urine samples and transferred to a slot in the device where levels of both estrone 3-glucuronide (E-3-G) and luteinizing hormone (LH) are measured by a patented immunochromatographic assay, utilizing an optical monitor. When a significant increase in the E-3-G level is detected, the fertility status is changed to 'unsafe'. After subsequent detection of the first significant rise of LH, the end of the fertile period is not signalled until 4 more days have elapsed. The system also stores and utilizes data on the individual's previous six menstrual cycles.

Efficacy information suggests a failure rate in consistent users no better than 6 per 100 woman-years, i.e. not as good as the best rates reported by perfect users of the symptom-thermal or multiple index methods. The advantages of the Personar are, however, that it is much simpler and quicker to use, with no charting and less abstinence required: a 'fertile' period lasting 8 days or less was signalled to 80% of users, and this is a definite improvement on the 10–12 days' abstinence usually demanded by the multiple index methods.

Male methods of contraception

Coitus interruptus

Coitus interruptus is the earliest form of reversible birth control (mentioned in the Book of Genesis and in Islamic texts), and is well described by its commonest euphemism, 'withdrawal' (before ejaculation, ensuring that all sperm are deposited outside the vagina). One study reported by the *Lancet* quotes 4% failure in the first year of 'perfect use'. Probable cause of failure results from partial ejaculation of a larger quantity of semen, either occurring a short while before the final male orgasm; or withdrawal during the latter rather than before it starts. It can therefore be useful to advise couples who want to continue using the method to use a spermicide as well.

Male condoms

The only proven barrier to the transmission of HIV, condoms are second in usage to the pill for those under the age of 30 and to sterilization above that age. 'Perfect use' results in a failure rate of 2%, with 'typical use' leading to 15% conceiving in the first year. The main reason for failure is either intermittent non-use or incorrect use, mainly through the escape of a small amount of semen either before or after the condom is in place for the main ejaculation, rather than because of condom rupture.

Vasectomy

Bilateral vasectomy is a safe and effective method of male sterilization. In the UK, around 23% of couples of reproductive age choose vasectomy as their method of contraception. As the sperm itself makes up a very small proportion of an ejaculate, vasectomy does not significantly affect its volume, appearance, texture or flavour. Two negative semen analyses (2–4 weeks apart and >8 weeks since the procedure) are required after the surgical procedure to ensure effectiveness. The incidence of chronic post-vasectomy pain is estimated to be less than 10%, depending on the severity of pain that qualifies for that particular study.

Vaginal methods of contraception

Female condoms

'Femidom' is the UK-marketed variety of female condom comprising a polyurethane sac with an outer rim at the introitus and a loose inner ring, the retaining action of which is similar to that of the rim of a diaphragm. It forms a well-lubricated (with silicone) secondary vagina, with an effectiveness rate in a 'perfect user' of approximately 5%. The condom is used near, or at the time, of intercourse, with the diaphragm and cap left in place for at least six hours after intercourse.

Caps and diaphragms

These create a vaginal barrier to sperm either at the cervix itself (caps of varying design) or in the upper

vagina (diaphragms). Their effectiveness is about 6 per 100 'perfect users', rising to 16 per 100 'typical users'. It is recommended that caps and diaphragms are supplemented with a spermicide (such as nonoxinol). Although invaluable as an adjunct to caps and diaphragms, and for some couples using coitus interruptus long term, spermicide used alone – whether as creams, jellies, pessaries or foams – is simply not acceptably reliable. However, good effectiveness has been reported in women whose fertility is already reduced.

The combined oral contraceptive (COC)

The COC is often seen as synonymous with contraception, and as such is commonly referred to as 'the pill'. The pill's mechanism of action is primarily to prevent ovulation, and secondarily to have contraceptive effects on the cervical mucus, and to impede implantation. While a variety of oral contraceptive pills exist, they all contain synthetic estrogen at 20–50 mcg (either ethinyloestradiol or menstranol) and a progestogen.

Two types of oral contraceptive pills exist: the monophasic or fixed pill, and the multiphasic or varying dose pill. The pattern of usage of the fixed pill depends on the exact preparation. In the most common regimen, pills are first taken on day 5 of a cycle, and continued for 21 days. The tablets are then either discontinued for 7 days, or a placebo is administered for 7 days, during which time withdrawal bleeding may occur. Multiphasic preparations contain tablets with varying amounts of estrogen and progesterone, which aim to simulate a more 'natural' hormonal pattern. The estrogen content increases from 30 mcg ethinyloestradiol in the first 6 days, to 40 mcg for the next 5 days, then back to 30 mcg for 10 days. The progestogen-levonorgestrel dose is increased from 50 mcg to 75 mg to 100 mcg in three stages.

The COC removes the normal menstrual cycle and replaces it with a cycle that is user-produced and based only on the end organ, i.e. the endometrium. As such, withdrawal bleeding has minimal medical significance, and can be deliberately postponed or made infrequent (e.g. tricycling – taking three consecutive packets, thereby reducing withdraw bleed frequency), and if it fails to occur, once pregnancy is excluded, poses no problem. Via occasional sensational reporting by the media, COCs have unjustly been a cause of worry for those clinicians prescribing it and women taking it. In 1995, the UK Committee on Safety of Medicines advised that COCs containing either gestodene or desogestrel were associated with twice the risk of venous thromboembolism compared with older products. However, this advice was based on their interpretation of three, then unpublished, studies. Following this announcement, a large proportion of women taking these so-called third generation COCs either discontinued use, or changed to other formulations. At the time, this led to a spike in unwanted pregnancies and terminations. In 1999, the Medicines Control Agency revised the estimate down to a 1.7-fold increase in risk.

The contraceptive benefits of COCs include their effectiveness and convenience, as well as the fact that they are not intercourse related. Another benefit of COCs lies in their reversibility. However, the non-contraceptive benefits of COCs may provide the principal indication for use of the method. These benefits include:

- Reduction of most menstrual cycle disorders: less heavy bleeding and therefore less anemia, and less dysmenorrhea; regular bleeding, the timing of which can be controlled; fewer symptoms of premenstrual tension overall; no ovulation pain
- Reduced risk of cancers of the ovary and endometrium, and possibly colorectal cancer
- Fewer functional ovarian cysts due to abnormal ovulation
- Fewer extrauterine pregnancies as normal ovulation is inhibited
- Reduction in pelvic inflammatory disease (PID)
- Reduction in benign breast disease
- Fewer symptomatic fibroids
- Probable reduction in thyroid disease, whether over- or under-active
- Probable reduction in the risk of rheumatoid arthritis
- Fewer sebaceous disorders (with estrogen-dominant COCs)
- Possibly fewer duodenal ulcers (not well established)
- Reduction in *Trichomonas vaginalis* infections
- Possible lower incidence of toxic shock syndrome
- Continuous use beneficial in long-term suppression of endometriosis
- No toxicity in overdose

Risks of COCs

Given the potential benefits of COCs, there are also risks associated with the majority of pharmaceutical

agents. For COCs, these risks include tumours (breast, cervical, liver, choriocarcinoma), cardiovascular (venous thromboembolism, VTE), and arterial diseases (acute myocardial infarction, hemorrhagic stroke and ischemic stroke). These risks must, however, be put into perspective, and not sensationalized. For example, the spontaneous incidence of a venous thrombotic event (VTE) in healthy, non-pregnant women (not taking any oral contraceptive) is about five cases per 100 000 women per year. The incidence in users of second-generation pills is about 15 per 100 000 women per year of use. The incidence in users of third-generation pills is about 25 cases per 100 000 women per year of use. The level of all of these risks of VTE increases with age and is likely to be increased in women with other known risk factors for VTE, such as obesity.

Tumour risk and COCs

There appears to be a slight (Odds Ratio of 1.24) increase in risk for breast cancer in women who use COCs. However, COC users can be reassured that the Odds Ratio of 1.24 signifies an increase of only 24% while women are taking the COC, diminishing to zero after discontinuation, over the next few years. Beyond 10 years after stopping, there is no detectable increase in breast cancer risk for former pill-users. The cancers diagnosed in women who use or have ever used COCs are clinically less advanced than those who have never used the pill, and are less likely to have spread beyond the breast. These risks are not associated with duration of use, the dose or type of hormone in the COC, and there is no synergism with other risk factors for breast cancer (e.g. family history). To put this in perspective, if 1000 women use the pill until the age 35, by age 45 there will be 11 cases of breast cancer. Importantly, however, only one of these cases is extra (pill-related), with the others having arisen in a control group of never-users.

Cervical cancer

COCs act as a co-factor for the Human papilloma virus (HPV) types 16 and 18, the principal carcinogen in cervical cancer, speeding transition through the stages of cervical intraepithelial neoplasia (CIN). In this respect it is similar to, but certainly weaker than, cigarette smoking.

Liver tumours

There is an increased relative risk of benign adenoma or hamartoma. However, the background incidence is so small (1–3 per 1 million women per year) that the COC-attributable risks are minimal.

Carcinomas of the ovary and of the endometrium

Both of these cancers are less frequent in COC-users. A protective effect can be detected in ex-users for up to 10–15 years. Suppression of ovulation in COC users, and of normal mitotic activity in the endometrium, are the accepted explanations of such findings.

Colorectal cancer

There are suggestive data, though the case is not yet fully proven, that the pill may also protect against this type of cancer.

Cardiovascular disease

There is no increased risk of hemorrhagic stroke (including subarachnoid hemorrhage) due to COC use in users under 35 years of age, unless there is also a risk factor such as hypertension (OR 10) or smoking (OR 3). The risk increases with age and this effect is magnified by current COC use, but with no effect of past use or long-duration use. However, there is a detectable increase in the Odds Ratio for the occurrence of ischemic stroke attributable to pill-taking in the range of 1.5 to a maximum of 2. However, much of this risk seems focused to within subpopulations who suffer from migraine with aura. Concerning the effect of dose/type of hormone, it is believed (though never proven) that modern low-estrogen pills help to minimize arterial risks. Whether the type of progestogen in the COC separately affects (as it can only do in those with risk factors) the arterial conditions above is still uncertain.

Progestogen-only pill (POP)

The mechanism underlying the action of POP is that it prevents ovulation in 50–60% of cycles, with the remainder relying mainly on progestogenic interference with mucus penetrability. This 'barrier' effect is readily lost, so that each daily tablet must be taken within 3 hours of the same regular time. The failure rate is 3.1 per 100 woman-years between the ages of 25

and 29, but this improves to 1.0 at 35–39 years of age and is as low as 0.3 for women over 40 years of age.

'Cerazette' is a relatively new POP that differs in its mode of action. Containing 75μg desogestrel and blocking ovulation in 97% of cycles, Cerazette exhibited a failure rate in a premarketing study of only 0.17 per 100 woman-years (in 'perfect' users). Twelve hours of 'leeway' in pill-taking have been approved before extra precautions are advised, unlike the 3 hours with the other POPs. Cerazette shares the medical safety, rapid reversibility but also, unfortunately, the tendency to irregular bleeding side effects and functional ovarian cyst formation of the old-type POPs.

Being estrogen-free, such products are exceptionally safe. There is no proven causative link with any tumour or with venous or (less certainly) arterial disease. The main side effect of POPs is irregular bleeding. FSH is not completely suppressed even during the amenorrhea, which is mainly caused by LH suppression. There is therefore enough follicular activity at the ovary to maintain adequate mid-follicular phase estrogen levels.

Injectables and implants

Injectables

In the UK, the only injectable currently licensed for long-term use is depot medroxyprogesterone acetate (DMPA) or Depo-Provera™, which has been given additional approval as a first-line contraceptive. WHO data indicate that DMPA-users have a reduced risk of cancer with no overall increased risk of cancers of the breast, ovary or cervix, and a fivefold reduction in the risk of carcinoma of the endometrium (relative risk 0.2). There are two injectable agents available: DMPA 150 mg every 12 weeks and Noristerat™ (Schering Health Care) (norethisterone enanthate) 200 mg every 8 weeks. Both are administered by deep intramuscular injection in the first 5 days of the menstrual cycle. Injections may also be given beyond day 5, with 7 days added precautions if it is near certain that a conception risk has not been taken.

DMPA is one of the most effective contraceptives among reversible methods with a 'perfect use' failure rate of 0.3%, and a failure rate of 3% following 'typical use' in the first year of use. It functions primarily by causing anovulation, backed by similar effects on the cervical mucus to the COC, as backup. The main side effects of DMPA are irregular, and sometimes prolonged, bleeding, alongside amenorrhea and potential hypo-estrogenism.

Contraceptive implants

Implants contain a progestogen in a slow-release carrier, made either of dimethylsiloxane as in Jadelle™ with two implants, or ethylene vinyl acetate (EVA) as Implanon™, a single rod. These are excellent examples of long-acting reversible contraceptives (LARCs) with the ideal 'forgettable' default state yet rapid reversibility. Implanon works primarily by ovulation inhibition, supplemented mainly by the usual sperm-blocking mucus effect. It is a single 40 mm rod, 2 mm in diameter, containing 68 mg of etonogestrel – the chief active metabolite of desogestrel – and so has much in common with Cerazette. It is medially inserted subdermally over the biceps in the upper arm, with local anesthesia. Implanon had the unique distinction of a zero failure rate in premarketing trials, though the 'perfect use' (= typical use) failure rate is now estimated as 5 in 10 000. Nearly all 'failures' that have been reported had involved insertion during a conception cycle or represented failures to insert.

Intrauterine contraceptives

There are currently two distinct types of intrauterine contraceptives, which include copper intrauterine devices (IUD), in which copper ions (the actual contraceptive) are released from a band or wire on a plastic carrier. The other type includes levonorgestrel-releasing intrauterine systems (LNG-IUS) which release progestogen as its mechanism of action.

Copper-bearing devices

Copper containing IUDs are safe (mortality 1:500 000) and effective. Their effectiveness is immediate and can be post-coitally applied (but not true of the LNG-IUS). Copper IUDs operate primarily by preventing fertilization, the copper ion being toxic to sperm. Their effectiveness when administered post-coitally shows that they can also act to block implantation. The problems and disadvantages of copper IUDs are relatively small when compared to hormonal methods. If a pregnancy does occur it is more likely to be an ectopic pregnancy (though absolute risk actually reduced in population terms). However, if intrauterine, then there is an increased risk of miscarriage as a result of

the IUD being present. At the time of IUD insertion, there is a risk of perforation of the uterus as well as of the bowel or bladder. Furthermore, at the time of insertion if there is a pre-existing STD in the cervix this may be transferred intrauterine, and result in a pelvic inflammatory disease.

The levonorgestrel-releasing intrauterine system (LNG-IUS)

The LNG-IUS I releases ~20μg per 24 hours of LNG from its polydimethylsiloxane reservoir, through a rate-limiting membrane, for its licensed 5 years (and longer). Its main contraceptive effects are local, through changes to the cervical mucus and utero-tubal fluid which impairs sperm migration, backed by endometrial changes impeding implantation. Its cumulative failure rate to 7 years is very low (1.1 per 100 women). Efficacy is not detectably impaired by enzyme-inducing drugs, and the systemic blood levels of LNG are under half of the mean levels in users of LNG POP. While the problems and disadvantages of the LNG-IUS are the same as with any intrauterine device, the amount of LNG in the blood is still enough to cause unwanted hormone-type side effects in some women; otherwise irregular light bleeding is the main problem.

Post-coital contraception

Three methods have now been shown to be effective contraceptives when used after unprotected sexual intercourse:

1. The insertion of a copper IUD
2. The combined oral emergency contraceptive (COEC) using LNG 500μg + EE 100μg repeated in 12 hours
3. The Levonorgestrel progestogen-only emergency contraceptive (LNG-EC) given as a stat dose of 1500μg LNG

The most widely used in the UK is the Levonorgestrel emergency contraception (LNG-EC). If it is administered at or before ovulation, then it acts by interfering with follicle development, either delaying or altogether inhibiting ovulation. If it is given later in a cycle, LNG-EC is capable of inhibiting implantation, but this seems to be the less effective of its mechanisms, so the failure rate tends to be higher for sexual exposures late in the cycle. LNG-EC has the greatest effectiveness, 99.6%, when treatment is begun within 24 hours of a single exposure. Reduced rates occur as a result of the main side effects of nausea and vomiting. There are virtually, in ordinary practice, no contraindications.

Copper intrauterine devices (IUDs)

Insertion of a copper IUD – not the LNG IUS – before implantation is extremely effective, through the toxicity of copper ions to sperm or by blocking implantation, preventing conception in approximately 99.9% of women. This means, after consultation with the woman, that insertion of an IUD may proceed in good faith, up to 5 days after the first sexual exposure (regardless of cycle length).

Female sterilization

Requests for sterilization are often due to individuals not wishing to continue using current reversible contraceptives. However, such requests are often based upon a lack of knowledge of current modern reversible options. Deferment, preferably avoidance, of surgery is often ideal, through careful discussion and explanation of alternatives, particularly long-acting reversible methods (LARCs).

It is often believed by individuals requesting sterilization that it is the most effective means of contraception. This is often not the case. For example, female sterilization using the 'Filshie clip' method (whereby fallopian tubes are blocked by insertion of a 'clip' over the tube) has an overall failure rate of around 0.2–0.3%, or a lifetime risk of three failures per 1000 procedures. Other types of tubal occlusion include: 'Falope Ring', a small silastic band placed around a loop of fallopian ring, which has a higher complication rate and failure rate than a Filshie clip. Another example is the 'Pomeroy Technique' which involves the removal of a portion of the fallopian tube. Again, there is a higher incidence of interoperative and postoperative bleeding, and is more difficult to reverse. A much lower late-failure rate can be quoted for vasectomies, namely one case in 2000 after negative semen testing at least 3 months after surgery.

The psychological sequelae for women undergoing female sterilization should not be underestimated. Considerable regret has been reported in 2% at 6 months and by 4% at 18 months, and postoperative psychiatric disturbance and dissatisfaction were largely associated with preoperative psychiatric disturbance. Higher rates of regret have been reported when sterilization is performed at times that are not,

except in rare special cases, now recommended, i.e. at termination of pregnancy or caesarean section, or immediately postpartum.

Sterilization, male or female, does not affect menstrual loss. However, if the contraceptive measure prior to the sterilization was COC, then the lighter regular withdrawal bleeds of the COC are replaced by normal menstruation. Therefore, counselling must include specific questioning about whether heavy bleeding or pain are, or were, problems during the woman's natural cycles, even if this relates to many years previously. This information is necessary to ensure that the right decision is made, which could be to use the LNG-IUS instead of either party being sterilized.

Further reading

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Chapter 16

Causes and investigations of male and female infertility

Tim Child

The aims of the chapter are to discuss causes of infertility and then to offer an evidence-based approach to diagnosis. The appropriate fertility treatments are discussed in Chapter 20.

About 85% of couples in the general population who are not using contraception and are having regular sexual intercourse will conceive within one year. Of those who do not conceive in the first year, about half will do so without treatment in the second year, giving a cumulative pregnancy rate by 2 years of at least 90%. Infertility is therefore generally defined as failure to conceive within 12 months of trying and affects around 15% of couples. Clearly the monthly chance of conception declines with increasing female age (Table 16.1), particularly from a woman's mid-30s, and so the rates of infertility will also increase in older populations. It is important to recognize that only a minority of couples attending a fertility clinic have 'infertility', i.e. an inability to conceive due to an absolute block to natural conception such as azoospermia or absent fallopian tubes. Most couples have a degree of 'subfertility' in that they continue to have a monthly chance of conception, albeit at a low rate.

The majority of women have menstrual cycles of 23–33 days' duration. Ovulation occurs around 14 (range 12–16) days before the first day of the subsequent period. Therefore, a woman with a regular 30-day cycle will generally ovulate around day 16. For natural conception to occur the woman needs to ovulate, have at least one patent fallopian tube plus an endometrium-uterus receptive to implantation, and the male partner's sperm function should be satisfactory. The main causes of infertility are based on abnormalities in these areas. Table 16.2 lists the causes and frequencies of infertility. The main cause headings are Female, Male and Unexplained.

Female infertility

Around 35% of couples will have infertility of primarily female origin. The causes include ovulatory dysfunction; fallopian, uterine or pelvic abnormalities including endometriosis; and advanced maternal age.

Ovulatory dysfunction

The World Health Organization (WHO) categorizes causes of amenorrhoea (absence of menstruation) due to ovulatory dysfunction as follows:

WHO Group I Ovulation disorders (hypogonadotrophic hypogonadism)

For normal ovulatory function to occur, there needs to be timely and coordinated FSH and LH drive from the anterior pituitary gland to the ovaries. Lack of hypothalamic-pituitary drive is termed hypogonadotrophic hypogonadism. Causes include low body weight or anorexia, excessive exercise, drugs, intracranial tumours or unknown (idiopathic).

WHO Group II Ovulation disorders (PCOS)

The most common cause of anovulation is the polycystic ovary syndrome (PCOS) which affects around 5% of women of reproductive age. For a diagnosis to be made, at least two out of the three factors listed in Table 16.3 must be present. PCOS causes around 85% of cases of oligo-amenorrhoea (irregular or absent periods). For most women with PCOS, the underlying etiology is a degree of peripheral insulin resistance. Raised serum insulin levels are associated with disrupted ovarian folliculogenesis, increased ovarian androgen production and reduced hepatic production of sex

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

Table 16.1 Cumulative conception rates following regular vaginal intercourse (Dunson *et al.*, 2004)

Female Age category	Pregnant after one year (12 cycles) (%)	Pregnant after two years (24 cycles) (%)
19–26	92	98
27–29	87	95
30–34	86	94
35–39	82	90

Table 16.2 Causes of Subfertility

Female Factor	35%
Tubal disease or other pelvic abnormality including endometriosis	
Ovulatory dysfunction	
Uterine abnormality	
Other including psychosexual	
Male Factor	**35%**
Unexplained	**30%**

Table 16.3 Diagnosis of polycystic ovary syndrome: the ESHRE/ASRM Rotterdam criteria

At least two of the following three criteria must be present for a diagnosis:
• Irregular menstrual cycles 6 weeks or more apart
• Polycystic ovarian morphology on ultrasound scan, i.e. at least one ovary to contain >=12 antral follicles or an enlarged ovary >=10 ml volume
• Clinical or biochemical hirsutism, i.e. acne or excess body hair, or raised serum androgens

hormone binding globulin. The raised serum androgen level (with reduced binding protein) leads to increased free androgens, which can cause hirsutism, and the impaired folliculogenesis causes anovulation. The condition is related to Type II diabetes mellitus, which is also a condition of peripheral insulin resistance. Obesity increases insulin resistance so women with PCOS who increase their weight will generally notice worsening of symptoms.

WHO Group III Ovulation disorders (ovarian failure)

The average age in the UK of the last period, termed the menopause, is around 51 years. Premature ovarian failure is when this occurs before the age of 40 and can be caused by pelvic surgery, endometriosis, chemotherapy or pelvic radiotherapy, genetic factors such as Turner syndrome (XO), or, very commonly, is unexplained.

WHO Group IV (hyper-prolactinaemia)

Raised prolactin levels, generally due to benign small pituitary tumours (adenomas), reduce GnRH drive to the pituitary. Certain drugs, such as some psychiatric medications, can also increase prolactin levels and have the same effect.

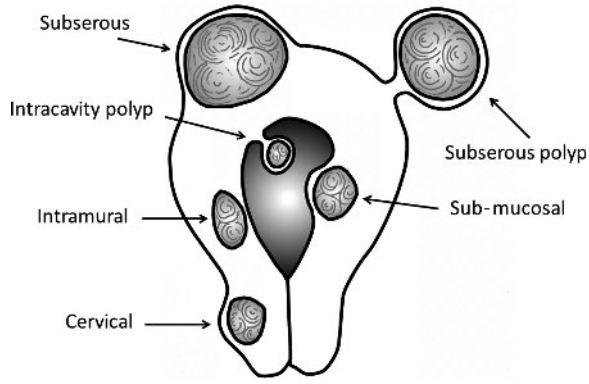

Figure 16.1 Types of uterine fibroids.

Tubal damage and uterine abnormality

The fallopian tubes can be damaged by ascending genito-urinary infection, particularly chlamydia, but also secondary to pelvic pathology such as endometriosis. Peritubal adhesions can be caused by previous surgery for pelvic pathology such as endometriosis, ovarian cysts, fibroids or bowel disease. A history of a previous tubal ectopic pregnancy indicates likely ongoing tubal damage, even in the opposite tube. The presence of tubal damage significantly increases the likelihood of tubal ectopic pregnancy following natural conception.

Even if oocyte fertilization does occur in the fallopian tube, abnormalities in the uterus can reduce the possibility of embryo implantation. Such abnormalities particularly include the presence of uterine fibroids. The closer a fibroid is to the endometrium and the greater the degree of endometrial disruption, the larger its negative effect is on implantation. Figure 16.1 illustrates types of fibroids. Sub-mucosal fibroids have the greatest detrimental effect, followed by intramural

types and then subserosal. The mechanism by which intramural fibroids not distorting the endometrial cavity affect implantation is not entirely clear. It is possible that factors released by the fibroid are the cause. Large fibroids may cause tubal blockage through a purely physical effect.

Endometriosis

Endometriosis is defined as the presence and proliferation of endometrial tissue outside of the uterine cavity, most commonly in the pelvis. Around 6–10% of women of reproductive age have endometriosis, most likely due to retrograde menstruation through the fallopian tubes with subsequent implantation and estrogen-driven growth of deposits. Using the revised American Society of Reproductive Medicine (ASRM) scoring system, there are four grades of severity of endometriosis; minimal, mild, moderate and severe. Endometriosis is classically associated with problems including pelvic pain and infertility, though many women are completely asymptomatic.

Infertility may be secondary to endometriotic adhesions disrupting normal tube-ovarian anatomy and so preventing pickup of the oocyte by the fimbriae. The presence of endometriosis cysts within the ovaries (called endometriomas) also reduces fertility through disrupting oocyte release and through increased possibility of peri-ovarian adhesions. While it is clear how severe endometriosis can cause infertility, as discussed above, the relationship between minimal amounts of disease and reduced fertility has been more difficult to explain. The peritoneal fluid of endometriosis patients contains significantly higher concentrations of inflammatory factors than is found in normal women. Some studies suggest that sperm function is negatively affected by such peritoneal fluid. Since the ampullae of the fallopian tubes, where oocyte fertilization occurs, are open to the pelvis and bathed in the abnormal peritoneal fluid, this may be one mechanism. Furthermore, recent work demonstrates that the endometrium within the endometrial cavity (i.e. eutopic endometrium) of women with endometriosis differs significantly from unaffected women. In particular, it is more inflammatory and demonstrates a degree of progesterone resistance and *in situ* estrogen production, all of which may be suboptimal for embryo implantation.

Psychosexual causes

Psychosexual causes of female infertility include problems such as vaginismus preventing penetration and completion of intercourse. There may be a history of sexual abuse. Embarrassment of psychosexual problems may lead to underreporting and underrecognition.

Advanced maternal age

The reduction in female fertility with increasing age is clear (Table 16.1). This is primarily due to the increasing proportion of aneuploid oocytes rather than the reduction in the actual number of gametes. An aneuploid oocyte will, following fertilization, result in an aneuploid embryo which is less likely to implant (so a lower chance of a positive pregnancy test) and, if it does implant, more likely to miscarry. These two outcomes together result in a significant decline in the chance of live birth as a woman goes through her late 30s and beyond per cycle of attempting natural conception or IVF. The problem is primarily oocyte rather than uterine, as illustrated by the observation that, with oocyte donation treatment, the success rate is related to the age of the donor, not the recipient.

Male infertility

For many men the cause of their abnormal semen analysis, particularly when the abnormality is mild, is unknown. Clearly, the presence of an abnormal semen analysis does not necessarily lead to an absolute inability for a partner to conceive, though the chance may be reduced.

Some aspects of male infertility are discussed in greater detail elsewhere in this book. In brief, causes of infertility/subfertility of male origin can include:

Primary testicular disease

- Genetic factors including Y chromosome microdeletions or Klinefelter (XXY) syndrome
- Testicular maldescent
- Testicular torsion
- Infection or trauma
- Testicular cancer and/or chemotherapy or radiotherapy (to the groin)
- Mumps or severe epididymo-orchitis

Obstructive male infertility

Obstruction can occur at any level of the male reproductive tract and can be due to congenital, inflammatory or iatrogenic causes. Men with cystic fibrosis have congenital bilateral absence of the vas deferens (CBAVD). Retrograde ejaculation into the bladder may occur secondary to neurological

complications of diabetes or surgical treatment of prostate disease. Erectile dysfunction can occur secondary to neurological, endocrine, vascular or psychological disorders.

Varicocoele

This is essentially a varicose vein(s) within the scrotum and is associated with an increase in local temperature, potentially affecting spermatogenesis. However, while varicocoeles are more commonly found in men with fertility problems, they are also not uncommon in 'fertile' men. Furthermore, treatment of the varicoele by surgery or radiological intervention has not been shown to improve live birth rates so should only be undertaken for men with symptoms from their varicocoele such as pain. Although there is some evidence that varicocoele repair may reduce levels of sperm DNA fragmentation, it is not currently known whether this improves the subsequent rate of conception or live birth.

Autoimmune causes

Some men develop autoantibodies to their sperm, particularly after scrotal surgery or trauma. The antibodies potentially affect sperm function and fertilization ability. However, it has not been demonstrated that measuring or 'treating' low to moderate antibody levels is of any benefit.

Endocrine causes

Absence of pituitary hormonal drive to the testes can be caused by hypogonadotrophic hypogonadism secondary to cranial tumours, drugs, trauma or conditions such as Kallman's syndrome or be unknown.

Environmental factors

These include exposure of the testes to chemicals, ionizing radiation or excessive heat.

Drugs

Prescribed drugs which can affect spermatogenesis include steroids, sulfasalazine, oral antifungals and opiates, and of course chemotherapy agents. Non-prescribed drugs, e.g. anabolic steroids for weight-lifting, marijuana, alcohol and tobacco, can also affect spermatogenesis and/or sperm function.

Unexplained infertility

A diagnosis of 'unexplained infertility' is rather a misnomer. There are clearly many unrecognized causes of subfertility. It is also possible that the cause is undiagnosed because of incomplete or poorly performed investigations. For example, many asymptomatic women correctly undergo hysterosalpingogram (HSG) testing for tubal patency rather than the more invasive laparoscopy procedure. However, without performing a laparoscopy it is generally not possible to diagnose conditions such as endometrioisis or peritubal adhesions. It is likely that the proportion of couples labelled as having 'unexplained' infertility will decline in years to come as we understand more about the pathophysiology of infertility and develop novel tests for detecting its causes.

Fertility investigations

For natural conception to occur the woman must ovulate, have at least one patent fallopian tube plus a receptive uterine environment, and sufficient quantity of motile sperm must be deposited in the vagina. The basic fertility history, examination and investigations are therefore focused on assessing these parameters.

History

The age and occupation of the woman and her partner are ascertained along with the duration of infertility. Couples should be asked how long it has been that they have not been using contraception. Some patients consider the duration of infertility to be how long they have been actively 'trying', i.e. checking for ovulation or timing intercourse, which is incorrect. Confirm that the couple are having regular intercourse and that there are no difficulties such as vaginismus, erectile dysfunction or severe premature ejaculation that interfere with this. If one partner has a job that involves night shifts or time away from home, then clearly this may be a factor.

The woman is asked about any previous pregnancies, time taken to conceive and method of conception, e.g. natural or with fertility treatment. What happened to each pregnancy; miscarriage, ectopic, premature delivery, natural or caesarean delivery and were there pregnancy complications such as preeclampsia or diabetes? She is asked about her menstrual cycle; regularity (suggestive of ovulation), pain (suggestive of endometriosis), heaviness (suggestive of fibroids) and details of any previous gynecological problems.

Any fertility treatments are then discussed, including details of the cycles and outcomes. If the patient has undergone treatment in another unit it is often useful to ask her to gain a copy of her old notes. She is asked

general questions including her past medical history (any previous acute or chronic illnesses including thyroid disease or diabetes), surgical history (any abdomino-pelvic surgery for, e.g. gynecological indications, burst appendix or bowel disease), and drug history, including prescribed, non-prescribed and illicit. How much does she drink and smoke? What is her weight, and if abnormal, is she managing or trying to correct it? Is she up-to-date with her cervical smears and has she had any abnormalities requiring treatment.

The male partner is asked about paternities and fertility treatments with previous partners. He is asked about his medical, surgical and drug history, and alcohol and tobacco use. In particular, had his testes descended by birth and/or has he required scrotal or hernia surgery or had significant groin trauma? Both partners are asked whether they have a history of sexually transmitted infection (STI), particularly chlamydia.

Examination

The body mass index (BMI) of the woman is calculated. It is debatable whether every female patient requires full, including vaginal, examination. A transvaginal ultrasound will yield more information from a fertility perspective than will a speculum and bimanual internal examination. Internal examination may be indicated if the woman has symptoms suggestive of endometriosis since this may not be diagnosed on ultrasound. There are some signs of endometriosis, such as nodularity behind the cervix or an immobile uterus, which can be detected on digital examination.

Men with severe oligo-azoospermia should undergo examination to assess testicular volumes, the presence of the vas deferens ducts and to exclude the presence of cysts, testicular tumours or a varicocoele. Small testes suggest testicular failure. The absence of one or both vas deferens suggests mutations in the cystic fibrosis genes.

Investigations

I Ovulation

Women with a regular menstrual cycle are most probably ovulating. While the only proof of ovulation is a pregnancy, a number of tests are used which assess the likelihood of ovulation.

i. Mid-luteal serum progesterone

This is the standard method of ovulation assessment. Progesterone is released into the circulation from the corpus luteum. Serum levels of >16 nmol/l are consistent with ovulation. The blood must be taken during the mid-luteal phase, around 7 days *before* the next menstruation, i.e. day 23 of a 30 day cycle. Women with irregular cycles may need to undergo repeated tests every 5–7 days so that the mid-luteal phase can be 'caught'. A low progesterone level in a woman with a regular menstrual cycle is very often due to mistiming of the test due to wrong instructions given by the attending doctor or misunderstanding by the patient.

Women with irregular menstrual cycles should be offered a blood test to measure FSH and LH, testosterone, prolactin and thyroid function. Normal levels of FSH suggest PCOS (levels of LH and testosterone may be raised, though not necessarily).

Low FSH levels suggest hypogonadotrophic hypogonadism which can be secondary to abnormal prolactin and/or thyroid levels, which must be excluded. Very often the low FSH picture is due to a very low BMI or excessive exercise. However, the possibility of a cranial tumour or congenital abnormality must be considered and excluded with an MRI.

High FSH levels suggest low ovarian reserve, i.e. premature ovarian failure. The ovarian reserve can also be assessed using ultrasound measurement of the antral follicle count (AFC) and serum measurement of anti-mullerian hormone (AMH). Ovarian reserve tests (FSH, AFC, AMH) are not of use in women who are ovulating regularly – they do not predict the chance of natural conception or the time to menopause. They are used to assess the cause of anovulation, or before IVF to assess the likely ovarian response and the chance of treatment cancellation. The tests do not predict the live birth rate during IVF.

ii. Urine LH sticks

Urine LH sticks can be purchased from a pharmacy and used to measure the concentration of LH in an early morning urine sample. Once the stick is 'positive' then ovulation *should* occur around 24–36 hours later.

iii. Basal body temperature (BTP)

Progesterone is thermogenic and, following ovulation, leads to an increase in basal body temperature of 0.5° C. Daily morning measurement and plotting of the temperature

may demonstrate the luteal phase increase suggestive of previous ovulation. However, the technique is labour intensive and the charts not always easy to interpret. BTP is therefore not a recommended investigation.

iv. Cervical mucus

Around the time of ovulation cervical mucus becomes stretchy, liquid and transparent. If allowed to dry on a slide and examined with microscopy a 'ferning' pattern will be seen. Following ovulation, due to the influence of progesterone, the mucus becomes viscous and thick. This is not recommended as a routine investigation.

v. Ultrasound follicular tracking

Transvaginal ultrasound is the standard method of assessing ovarian follicular development. Serial ultrasound scans from around day 10 of the menstrual cycle will show a preovulatory follicle to have an increasing diameter. Ovulation occurs at a follicular diameter of around 20 mm. After ovulation the corpus luteum is seen to contain shadows consistent with clotted blood. While a baseline transvaginal ultrasound is a useful investigation for assessing the female pelvic organs, it is too labour intensive and expensive a tool to be used for routine follicular tracking apart from when a patient is receiving ovarian stimulation treatment.

II Normal female reproductive tract

Tubal patency can be assessed using a hysterosalpingogram (HSG), a laparoscopy and dye, or hysterocontrast-ultrasonography (HyCoSy). However, since all of the tests are invasive, they should only be performed if the findings are going to affect patient management. For example, there is little point in performing tubal patency testing if the male partner has severe oligospermia requiring IVF-ICSI. It is therefore normal practice to confirm the results of a semen analysis first.

i. Hysterosalpingogram (HSG)

The procedure is performed following insertion of a vaginal speculum and visualization of the cervix. A plastic or metal catheter is passed trans-cervically into the lower part of the uterine cavity. No anaesthetic is required and the patient is able to see the images on a monitor. A radio-opaque dye is injected through the catheter and a series of X-ray images taken of the pelvis. In a normal pelvis the uterine cavity is seen to first fill, followed by the fallopian tubes, before the dye spills from the fimbriae into the pelvic cavity. This is often reported as 'fill and spill'. The test also excludes conditions such as intrauterine scarring (Asherman's syndrome), uterine anomalies such as bicornuate or septate uterus and a distorted endometrial cavity due to fibroids. Cramping pains are very common during the HSG as the dye expands the cavity, particularly when there is tubal blockage, and can be diminished by taking oral analgesia pre-procedure. The HSG should be performed after menstruation but before ovulation to avoid exposing an implanting embryo to both radiation and the dye. The sensitivity of HSG for correctly diagnosing tubal patency is over 80%. Sometimes the seal at the cervix may be poor, leading to backflow and insufficient dye pressure to pass through the fallopian tubes. In addition, the smooth muscle of the fallopian tubes can spasm, preventing passage of dye and falsely suggesting tubal blockage. Advantages include the avoidance of general anesthesia or surgical incisions with the accompanying risks. Other risks include the possibility of introducing infection into the pelvis; consequently women should be screened for chlamydia or receive oral antibiotic cover if unscreened. If the HSG is abnormal, then a laparoscopy and dye may be performed.

ii. Laparoscopy and dye

'Lap and Dye' is the gold-standard investigation of the female pelvis. The procedure is performed under a general anesthetic and involves insufflation of the abdomen by CO_2 gas followed by inspection of the pelvis via a telescope inserted through the umbilicus (Figs 16.2 and 16.3). The pelvic structures are examined and blue dye is then injected via a catheter through the cervix. The fallopian tubes are seen to 'fill and spill' in a normal pelvis. Advantages of a laparoscopy and dye include the ability to visualize the external surface of the uterus and tubes directly and to look for other causes of infertility such as endometriosis, adhesions or fibroids. If present, and not too severe, then endometriosis and adhesions can be removed during the same procedure ('see and treat'), though further discussion is generally required before surgically treating severe endometriosis or adhesions or removing fibroids because of the extra risks involved. The risks of a

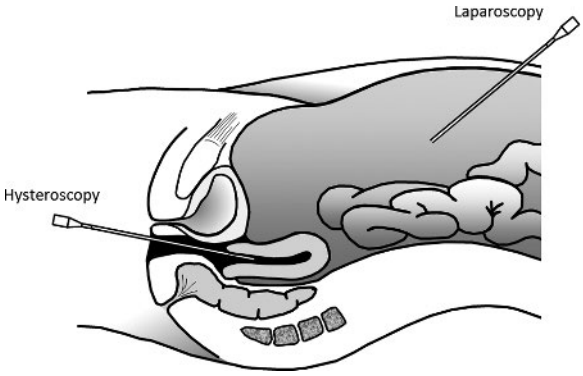

Figure 16.2 Diagram of a laparoscopy and hysteroscopy.

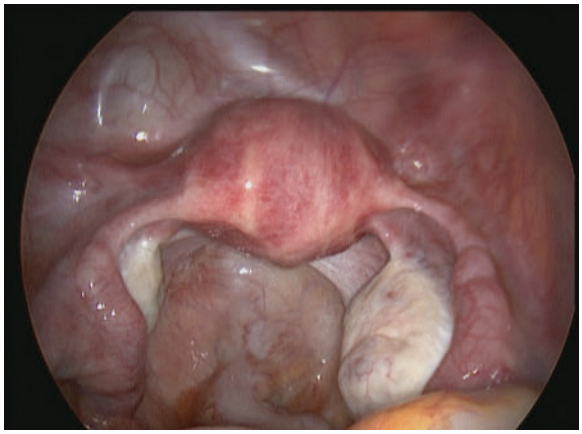

Figure16. 3 Laparoscopic view of a normal pelvis. The uterus, tubes, and ovaries are seen from above.

lap and dye include the (low) risk of anaesthesia and a risk of 2–3/1000 of causing damage to intra-abdominal structures such as the bladder, bowel, blood vessels or ureters.

iii. HyCoSy (Hystero-Contrast-Ultrasonography)

This is a similar technique to HSG in which an ultrasound opaque liquid is injected via a trans-cervical catheter into the uterine cavity. Using a transvaginal ultrasound (rather than X-ray) the fluid is seen to pass through the uterine cavity into the fallopian tubes and, if patent, pass into the pouch of Douglas. Advantages of HyCoSy compared to HSG include the avoidance of radiation and the ability to visualize ultrasonographically other pelvic structures such as the ovaries and the uterine myometrium allowing the diagnosis of fibroids. A disadvantage is the lack of hard images for later independent assessment; all that can be recorded is the fluid collecting in the pelvis. HSG also gives a better indication of the degree of any abnormality within the uterine cavity.

iv. Ultrasound scan

Transvaginal ultrasound has become an indispensable tool in infertility investigation and management. Ultrasound is used to assess uterine anatomy to search for abnormalities such as endometrial polyps, fibroids or uterine congenital anomalies such as septate or bicornuate shapes. The ovary is examined to diagnose the presence, size and type (e.g. endometrioma, simple or dermoid cyst) of ovarian cysts and also to make an assessment of 'ovarian reserve' through measurement of the antral follicle count (AFC) and ovarian volume. Sometimes the presence of adhesions can be suspected when an ovary is seen to be deep in pelvis, often stuck to the posterior wall of the uterus, and is immobile on moving the transvaginal probe. Pain during the ultrasound procedure may also suggest the presence of adhesions and/or endometriosis. Collections of fluid in the adnexae may be within the fallopian tube (hydrosalpinx) or within pelvic adhesions. If a hydrosalpinx is confirmed, then removal of the affected tube before IVF is often undertaken to improve success rates.

Women who are not known to have comorbidities (such as pelvic inflammatory disease, previous ectopic pregnancy or endometriosis) should be offered HSG or HyCoSy to screen for tubal occlusion because this is a reliable test for ruling out tubal occlusion, and it is less invasive and makes more efficient use of resources than laparoscopy. Women who are thought to have comorbidities should be offered laparoscopy and dye so that tubal and other pelvic pathology can be assessed at the same time.

III Sperm

i. Semen analysis

Semen is examined using the 2010 World Health Organization criteria as per Table 16.4. The revised values are based on the 5th centile of a fertile population. The man should be asked to abstain from ejaculation for 2–5 days beforehand. Where the analysis is performed well there is a good correlation between the measures of semen quality obtained (count, motility and morphology) and the probability of conception. If abnormal, the test

Table 16.4 Reference values for minimum semen quality compatible with normal fertility according to World Health Organization 2010 values

Variable	Value	Unit
Volume	1.5	ml
Concentration	15	x 10^6 per ml
Total number	39	x 10^6 per ejaculate
Progressive motility	32	% grades a+b
Morphology	4	% normal forms
Vitality	58	% alive
pH	7.2	pH units
White blood cells	< 1.0	x 10^6 per ml

should ideally be repeated 3 months after the initial analysis to allow time for the cycle of spermatozoa formation to be completed. However, if a gross deficiency (azoospermia or severe oligospermia) has been detected, the repeat test should be undertaken as soon as possible.

ii. Hormones

In cases of azoospermia, a serum profile should be checked for FSH, LH, testosterone, prolactin and thyroid hormones. Very low levels of FSH and LH with low testosterone levels are consistent with hypogonadotrophic hypogonadism. Normal levels of FSH, LH and testosterone suggest blockage (obstructive azoospermia) somewhere along the efferent system. High levels of FSH and LH along with low levels of testosterone suggest primary testicular failure. Abnormal prolactin and/or thyroid hormone levels can lead to a hypogonadotrophic picture, as can cranial congenital abnormalities or tumours, which should be excluded by an MRI.

iii. Genetic investigations

Men with severe oligospermia (count < 5 million/ml) or azoospermia should undergo karyotype and cystic fibrosis (CF) gene analysis. The commonest genetic cause of azoospermia is Klinefelter syndrome (XXY). The presence of chromosomal translocations can also result in oligo-azoospermia. Mutations in the CF gene are associated with bilateral absence of the vas deferens, or oligospermia in the absence of other features of cystic fibrosis such as lung disease.

More recently analysis of sperm aneuploidy and DNA fragmentation has been possible. It is not clear the extent to which the results of these tests can be used to dictate subsequent management.

iv. Antisperm antibodies

Antisperm antibodies (ASA) may be present in the ejaculate due to previous trauma or surgery. Routine measurement of antibody types and levels is not indicated. If antibodies are present to the extent that agglutination affects sperm function, then the semen analysis result, without the aid of antibody testing, is likely to dictate subsequent management. There are no drugs shown to improve fertility in men with ASA.

Conclusion

By undertaking investigations in a logical manner, the majority of couples with fertility problems will have a diagnosis made and can move on to the appropriate treatment. Since investigations take time, cost money and may have risks, it is important to consider the particular situation of the couple. It may be appropriate for some couples to move on to definitive treatment such as IVF without undergoing complete fertility investigations, for instance after 10 years of infertility where the woman is 39 years of age. Other patients prefer extensive investigation to feel that they fully understand their situation and to confirm that nothing more could be done prior to assisted conception or stopping treatment.

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Chapter 17

Treatment of male and female infertility

Tim Child

Once a couple experiencing fertility problems have undergone appropriate and timely investigations then, in the majority of cases, a diagnosis can be made. A minority will have the rather unsatisfactory diagnosis of exclusion, 'unexplained infertility'. A treatment plan can then be made. The patients should attend the consultation together.

Pre-pregnancy counselling

Women who are trying to become pregnant should be informed that drinking no more than one or two units of alcohol once or twice a week, and avoiding episodes of intoxication, reduce the risk of harming a developing fetus. Men who drink up to three or four units of alcohol per day are unlikely to affect their fertility. Excessive alcohol intake can affect semen quality.

Women who smoke should be informed that this is likely to reduce their fertility and should be offered referral to a smoking cessation programme. Passive smoking may also affect female fertility. While there is an association between male smoking and reduced semen quality, the impact of this on fertility is unclear.

Dietary supplementation with folic acid before conception and up to 12 weeks' gestation reduces the risk of having a child with a neural tube defect. The recommended dose is 0.4 mg per day, though for women with diabetes, on anti-epileptic medication or who have previously had a child with a neural tube defect, a dose of 5 mg per day is recommended.

A female body mass index (BMI) over 29 is associated with a longer time to conception and a higher rate of miscarriage. Women who are not ovulating, and who have a BMI over 29, are likely to improve their chances of conception by losing weight. Similarly there is a correlation between male obesity and reduced fertility. Women with low BMI of less than 19 and who have irregular or absent menstruations are likely to improve their fertility by increasing their weight.

While there is an association between elevated scrotal temperature and reduced semen quality, it is not clear whether wearing loose-fitting underwear improves fertility.

Some occupations involve exposure to hazards that can reduce male or female fertility, and appropriate advice offered.

A number of prescription, over-the-counter and recreational drugs interfere with male and female fertility and so should be enquired about, and appropriate advice given.

Vaginal sexual intercourse every 2–3 days through the cycle optimizes the chance of conception.

For couples with a diagnosed cause of infertility, the treatment will depend on the cause.

Ovulation disorders

Following investigation, the cause of ovulatory dysfunction should be classified (see Chapter 20):

WHO Group I Ovulation disorders (hypogonadotrophic hypogonadism)

Women with WHO Group I anovulatory infertility can improve their chances of conception and an uncomplicated pregnancy by moderating high exercise levels and increasing the body weight if the BMI is less than 19. Pulsatile subcutaneous administration of gonadotrophin releasing hormone via a pump is a physiological and successful way of inducing monoovulatory cycles. However, the need to wear the pump constantly limits the use of this technique. Ovulation induction with once daily sub-cutaneous gonadotrophin injections for two weeks or so is more commonly used. The absence of endogenous LH pituitary

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

production means that a gonadotrophin with LH activity should be used in addition to FSH. The ovarian response needs to be closely monitored with ultrasound to reduce the risk of hyperstimulation and multiple pregnancy. An hCG injection will be required to induce ovulation, followed by timed intercourse.

WHO Group II Ovulation disorders (PCOS)

Women with WHO Group II ovulation disorders who are overweight should be encouraged to normalize their BMI. This may promote spontaneous ovulation or increase the response to ovulation induction drugs and also reduce risks during pregnancy.

Clomifene citrate

The anti-estrogen clomifene citrate has for decades been the first-line ovulation induction drug for PCOS. Clomifene blocks the estrogen feedback from the ovaries to the pituitary and hypothalamus, 'tricking' the pituitary into releasing more FSH which may be sufficient to result in follicular development. Clomifene is taken as a tablet, usually at an initial dose of 50 mg once daily for 5 days from day 2 of the menstrual cycle. Side effects include headaches and visual disturbances. If these occur then clomifene must be stopped and an alternative treatment used. The most important side effect is a 10% multiple pregnancy rate, nearly always twins, though the author has seen two sets of quadruplets following clomifene treatment. It is good practice to offer ultrasound monitoring in the first cycle to recognize the development of too many dominant follicles, cycle cancellation and dose reduction in the next cycle. Failure to respond at all to clomifene ('clomifene resistance') leads to a step increase in the clomifene dose each cycle to a maximum of 150 mg daily. If still clomifene resistant even at the maximum dose then second-line treatments as discussed below are used. Clomifene is licensed for a maximum of six cycles of treatment. Very prolonged use (over 12 months) has been linked with a possible increase in the risk of developing ovarian cancer.

Metformin

As discussed in Chapter 20, PCOS appears to be a condition of insulin resistance. Obese women with anovulatory PCOS, who reduce their weight by 5% or more, will also reduce their insulin resistance and may begin to ovulate spontaneously. If not then the insulin sensitizing agent metformin can be used. Metformin is taken in multiple doses every day, unlike clomifene which is only taken for 5 days per cycle. Metformin's side effects include nausea, vomiting and other gastrointestinal disturbances. It does not promote weight loss.

A number of RCTs have compared clomifene against metformin against combined clomifene and metformin for first-line ovulation induction in women with PCOS. A recent NICE (National Institute for Health and Clinical Excellence) meta-analysis suggests similar cumulative live birth rates with the different treatments. An advantage of metformin is that it promotes mono-ovulation so there's no need for ultrasound follicular tracking. In addition, metformin may normalize testosterone levels and consequently reduce hirsutism, thus having additional non-fertility benefits. The need for daily multiple doses and the gastrointestinal side effects are disadvantages. The main disadvantage of clomifene is the multiple pregnancy rate. Hence, the options should be discussed with women to enable them to make an informed choice.

Women who are clomifene resistant can undergo one of the following second-line treatments: laparoscopic ovarian drilling, gonadotrophin therapy, or combined treatment with clomifene and metformin if not already used first line. Success rates appear similar between the options.

Laparoscopic ovarian drilling (LOD)

During a laparoscopy the ovaries are each 'drilled' using a diathermy electrical current for a few seconds in multiple places. This technique has replaced the now obsolete 'wedge-resection' procedure. An advantage of LOD is that other pathology such as endometriosis or adhesions can be diagnosed and treated during the same procedure. Tubal patency can also be tested ('lap and dye'). Also, if successful, then the resulting mono-ovulation is consequently not associated with an increased risk of multiple pregnancy or the need for ultrasound follicular tracking. Furthermore, if successful, the effect can last for many years after a single procedure. Disadvantages include the need for surgery and the associated risks of anesthesia and intra-abdominal organ damage. There is a risk of causing the formation of peri-ovarian adhesions which could reduce fertility. Rarely, premature ovarian failure has been reported secondary to the ovarian trauma. It is not clear how LOD has its effect. The 'drilling' disrupts the ovarian stroma and appears to reset the milieu allowing folliculogenesis to commence.

Gonadotrophin therapy

Gonadotrophins are administered by daily subcutaneous injection and are either recombinant or urinary derived. Disadvantages of gonadotrophin treatment include the need for frequent ultrasound follicular tracking and the risk of multiple pregnancy, which occurs with rates of up to 20% or more. The multiple rate depends on the threshold maximum 'safe' follicle number set by the doctor for inducing ovulation. For instance, some clinics will cancel the treatment cycle if there are four or more mature follicles, which will clearly mean there is a triplet risk if all three dominant follicles ovulate.

The use of 'low-dose step-up' gonadotrophin regimes for ovulation induction in PCOS patients results in multiple pregnancy rates of < 10% (i.e. similar to clomifene). The gonadotrophins are started at a low dose of between 25 to 75iu and held at that dose for 10 days before the first ultrasound monitoring scan. If a dominant follicle >10 mm diameter has developed, then the same dose is continued for a few days. A further scan is arranged to confirm the presence of a preovulatory follicle, at which time an hCG trigger is given to induce ovulation followed by timed intercourse. If on the initial day 10 scan there is no follicular response, then the gonadotrophin dose is increased by a small amount and the scan repeated every seven days and the dose increased until a follicular response is achieved and ovulation can be induced.

Meta-analysis suggests that patient satisfaction and cumulative success rates are similar between LOD and gonadotrophin therapy. The 'one-stop' nature of LOD, the avoidance of ultrasound monitoring, daily injections and multiple pregnancy risk are clear advantages. However, many women prefer to avoid surgery and to move on to more immediate treatment using gonadotrophins rather than wait and see whether ovulation results after LOD.

Assisted conception

The third-line treatment for infertility due to PCOS is assisted conception, the standard method being IVF. In summary, IVF involves gonadotrophin ovarian stimulation followed by transvaginal oocyte retrieval, in vitro oocyte fertilization and culture, and transcervical embryo transfer. In long-protocol IVF, the hypothalamo-pituitary axis is suppressed by administration of a GnRH-agonist for a few weeks before commencing gonadotrophins. In short-antagonist protocol IVF, a GnRH-antagonist is commenced around day 5 to 7 of gonadotrophin stimulation without prior suppression. Live birth rates are similar between long- and short-antagonist protocol IVF for women with PCOS. However, the risk of developing ovarian hyperstimulation syndrome (OHSS), the main health risk to women undergoing IVF, is significantly lower with the short-antagonist protocol. If long-protocol IVF is used, then co-treatment with metformin tablets will also significantly reduce the risk of developing OHSS. It is not known whether the use of metformin co-treatment during short-antagonist IVF is of additional benefit.

Risk factors for developing OHSS during IVF include younger age (< 33 years), previous OHSS and the presence of ovaries of polycystic morphology. OHSS can be mild, moderate or severe. Mild or moderate OHSS may cause 'only' discomfort, nausea and diarrhea. However, severe OHSS is potentially, though rarely, fatal and requires hospital admission for intravenous rehydration and thromboprophylaxis, along with close monitoring of fluid balance and blood haematology, clotting and biochemistry factors. The rate of severe OHSS is about 1% of all IVF cycles. Women with PCOS undergoing long-protocol IVF have a severe OHSS rate of 2–10%; this is reduced to 1–3% with the use of metformin co-treatment or by using a short-antagonist protocol. A number of other strategies are also available to reduce the risk of developing OHSS and are reviewed elsewhere.

The only way of absolutely avoiding the risk of developing OHSS is to not stimulate the ovaries. Oocyte in vitro maturation (IVM) involves the transvaginal aspiration of immature oocytes from unstimulated ovaries, followed by their in vitro maturation and fertilization. Embryos are then cultured in vitro and transferred trans-cervically. IVM is fully reviewed in another chapter. IVM is most successful for younger women with ovaries of polycystic morphology (i.e. two of the main risk factors for OHSS). While clearly there is zero risk of developing OHSS in a woman undergoing IVM, and the treatment is very 'easy' and acceptable from a patient perspective, the success rate is currently significantly less than IVF, which limits its desirability.

WHO Group III Ovulation disorders (ovarian failure)

Anovulation due to ovarian failure is detected by high levels of FSH, or low levels of AMH or a low AFC. The

woman may have a family history of premature ovarian failure, a personal history of chemo-radiotherapy or removal of ovarian tissue, for example while removing endometriotic cysts, or have a genetic disorder such as Turner syndrome.

There are no drugs that can be given to boost fertility in cases of ovarian failure. The treatment is oocyte donation or moving on from fertility treatments to other options such as adoption or accepting childlessness. Potential recipients of donor oocytes are offered counselling regarding the physical and psychological implications of treatment for themselves and their potential children. In the UK, children born from gamete (oocyte and sperm) or embryo donation are able to trace the donor from the age of 18 years. Oocyte donors are screened for both infectious and genetic diseases and undergo a full stimulated IVF cycle. Their oocytes are collected and fertilized in vitro with the recipient's partner's sperm. The recipient's endometrium is prepared with exogenous oestrogen and progesterone in coordination with the donor's cycle and embryo transfer then takes place. The success rate is related to the age of the donor. This must be taken into account when deciding how many embryos to transfer. Pregnancy rates of around 50% per cycle are common.

WHO Group IV (hyperprolactinaemia)

Women with ovulatory disorders due to hyperprolactinaemia should be offered treatment with a dopamine agonist such as bromocriptine under the care of an endocrinologist.

Tubal and uterine disease

Tubal damage

Hysterosalpingogram, HyCoSy or laparoscopy may demonstrate the presence of tubal disease. If one fallopian tube is patent then the cumulative chance of conception is satisfactory and no particular treatment is required. If both tubes are blocked then treatment options depend on the position of the block (proximal vs. distal) and severity of the disease.

Mild distal (at the fimbrial end) tubal disease can be treated by laparoscopic fimbrioplasty in which the blocked ('clubbed') tubal ends are surgically opened and 'flowered-back'. There is little role for this if the rest of the tube is damaged, particularly if a hydrosalpinx is present. Even if tubal patency results, the patient must be warned that the blockage may recur and that, if she conceives, she is at significantly increased risk of developing a tubal ectopic pregnancy. Early ultrasound in pregnancy is required to confirm an intrauterine position. If the disease is more severe or involves the whole tube, then surgery is unlikely to be of benefit.

IVF was developed as a treatment for tubal disease and remains the most successful form of therapy. The presence of an ultrasound-visible hydrosalpinx is associated with a halving of the IVF success rate due to leakage of the fluid into the uterine cavity. Removal of the affected tube(s) restores the IVF success rate to what it would have been if there were no hydrosalpinx (Figure 17.1). Some women with a hydrosalpinx note a watery brown vaginal loss off and on throughout the menstrual cycle. Ultrasound can often demonstrate the fluid within the endometrial cavity. The hydrosalpinx fluid contains embryo-toxic substances. There is also the purely mechanical effect of the fluid flushing the embryo. Some women may, however, be resistant to the suggestion, particularly with bilateral hydrosalpinges, that their fallopian tubes are removed, leaving them permanently sterile. If there are extensive adhesions in the pelvis, then removal of the tubes can be difficult, so sometimes a clip is applied laparoscopically at the cornu, where the tube enters the uterus, to prevent fluid leakage into the endometrial cavity. A newer hysteroscopic technique involves insertion, via the uterine cavity, of an implant through the tubal ostia into the proximal part of the tube ('Essure'). The product was developed as a form of contraception and is unlicensed for this indication.

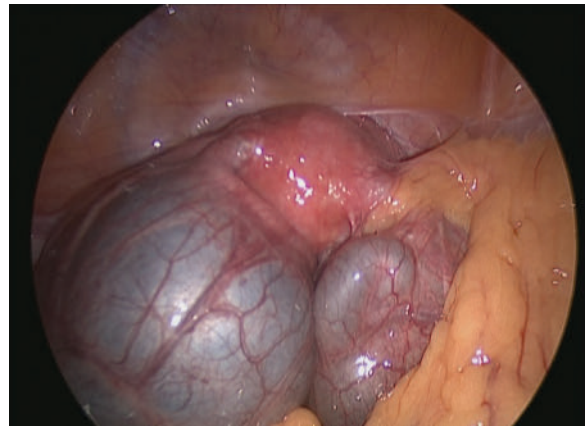

Figure 17.1 Laparoscopic view of bilateral hydrosalpinges.

Treatments such as ovulation induction or IUI are inappropriate for women with tubal disease.

Intrauterine adhesions

An uncommon cause of amenorrhea is extensive intrauterine adhesions ('Asherman's syndrome') usually due to endometrial curettage for a miscarriage or retained placental tissue after delivery. The basal endometrial layer is damaged to the extent that proliferation and endometrial thickening does not occur and so neither does menstruation, despite there being ovulatory cycles. Sometimes less extensive intrauterine adhesions are found in women who are menstruating but who have fertility or recurrent miscarriage problems. The presence of intrauterine adhesions can be suspected on ultrasound scan but is confirmed on HSG or hysteroscopy. Hysteroscopic resection of the adhesions is undertaken and an intrauterine coil left in place for a month to try to reduce adhesion reformation. Often, since the basal endometrial layer is damaged, the result is relatively poor. Under these circumstances surrogacy may be required.

Fibroids (leiomyomas)

Fibroids which are distorting the endometrial cavity may be removed, a procedure called myomectomy. The method of removal depends on the site and size of the fibroid(s). Fibroids within the endometrial cavity are removed using a hysteroscope inserted through the cervix under general anaesthesia (Transcervical Resection of Fibroid, TCRF). The cavity is irrigated with glycine and electrical current passed through a semi-circular loop which is used to cut away the fibroid in strips for removal through the cervix. The same method is used for sub-mucosal fibroids of up to 3 cm diameter. Risks of TCRF include perforation of the uterine wall and intrauterine adhesion formation. Larger fibroids distorting the endometrial cavity are removed abdominally, preferably by laparoscopy rather than open surgery. Risks of myomectomy, by any route, also include bleeding requiring blood transfusion or further surgery, and rarely, to save a life, hysterectomy.

While it is generally accepted that myomectomy is appropriate for fibroids distorting the endometrial cavity, the situation for intramural fibroids that are not distorting the cavity is not so clear. It is accepted that such fibroids do reduce the implantation rate; however, whether removal of the fibroids improves the rate is not known since sufficiently powered RCTs have not been undertaken. Certainly if the woman has symptoms attributable to her fibroids, such as heavy menstrual bleeding or bladder-bowel pressure symptoms, then surgery is probably indicated.

Endometriosis

Laparoscopic removal of minimal to mild endometriosis is associated with a statistically significant increase in the rate of natural conception and so should be offered. The endometriosis is removed by cutting away using scissors or laser, or is ablated using electric diathermy.

Laparoscopic removal of endometriotic ovarian cysts (cystectomy) is associated with an increase in the subsequent rate of natural conception. There are two methods of treating cysts. The first step is to open and drain away the 'chocolate' cyst fluid within the cyst. The wall can then either be stripped away or an attempt made to ablate it. Stripping has the advantage of allowing the tissue to be sent for histopathological analysis. Occasionally cysts thought to be endometriotic are found to be malignant or borderline in character. Stripping of the cyst wall is also associated with a higher natural cumulative conception rate and a lower chance of cyst recurrence. However, cystectomy can cause further damage to the ovary, which may reduce the response to ovarian stimulation during IVF.

It is unclear whether endometriomas should be removed prior to IVF. No sufficiently powered RCTs have been undertaken. Cystectomy does not improve the ovarian response to stimulation (and, if the ovary is further damaged, may have the opposite effect) (Figs 17.2 and 17.3). It may improve ovarian accessibility for transvaginal oocyte recovery. Certainly during oocyte recovery it is important to avoid passing the needle into an endometrioma, as this can lead to pelvic infection and possible ovarian abscess formation. Surgery may be required to treat a pelvic abscess and the ovary may be permanently damaged. If an endometrioma is entered during oocyte recovery, intravenous antibiotics are given.

Women with moderate to severe endometriosis may benefit from surgical removal of disease and adhesions to improve their fertility and/or pain symptoms, though no randomized studies have been undertaken to test this hypothesis. However, very often the most appropriate treatment is IVF. Prolonged GnRH-analogue down-regulation for two

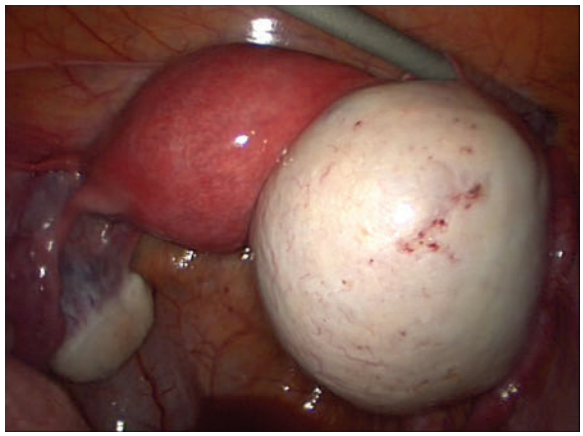

Figure 17.2 Laparoscopic view of a cyst within the right ovary.

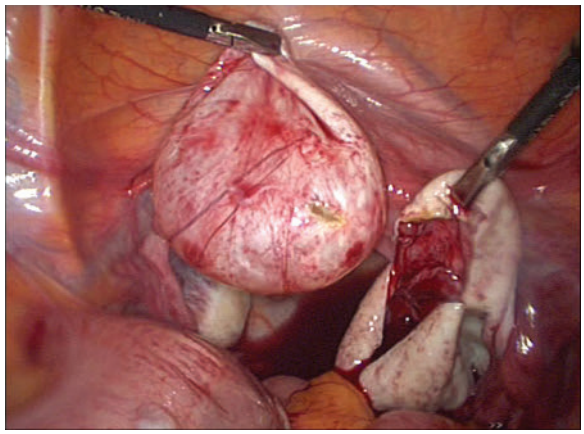

Figure 17.3 The cyst has been stripped from the right ovary.

or three months before long-protocol IVF in women with severe endometriosis has been shown to improve the live birth rate, possibly through improving endometrial receptivity. Whether or not the same outcome can be achieved by using prolonged oral contraceptive pill pretreatment is not currently known.

Absent or severely abnormal uterus

Women may have an absent uterus due to a congenital abnormality such as Rokitansky syndrome or following hysterectomy for malignancy. The uterus may be severely abnormal due to extensive fibroids or endometrial abnormalities such as Asherman's syndrome. If the woman's ovaries are still functioning then she can undergo a stimulated IVF cycle, produce embryos with her partner's sperm and have the embryos transferred into a 'host surrogate'. If the woman does not have functioning ovaries then her partner's sperm can be used to inseminate the surrogate, known as 'straight surrogacy'. Clearly there are a number of legal and ethical issues surrounding surrogacy, though it is a successful (and only) form of treatment for many couples.

Unexplained infertility

For couples with unexplained infertility there is no place for ovarian stimulation treatment using oral drugs such as clomifene citrate, or the lesser used drugs tamoxifen, anastrozole or letrozole. Patients, and doctors, often presume that the boost clomifene gives to ovulation, potentially resulting in multiple ovulation, will increase the chance of conception in women who are already ovulating spontaneously. A number of studies have shown this not to be the case. The explanation may be that the anti-estrogenic effects of clomifene have deleterious effects at the endometrium.

Expectant management for a period of time may be appropriate. This involves giving advice on lifestyle factors, as initially described in this chapter, and excluding pathology that would require immediate recourse to fertility treatment. It is helpful to agree on a time frame with the couple, for instance to continue trying naturally for another six months before review and potentially moving on to active treatment. It is also helpful for the couple to have access to the fertility clinic nurse, counsellor or dietician for consultations. Expectant management is often also appropriate for couples with a diagnosis of minimal-mild endometriosis or mild male factor when there continues to be a reasonable monthly chance of conception for infertility durations of up to 2 or 3 years. Expectant management may be the only option for couples who cannot afford IVF or where the woman's ovarian reserve is so diminished (despite still ovulating regularly) that IVF is not possible.

Intrauterine insemination (IUI) has been used as a treatment for unexplained fertility for many years. There is no evidence that unstimulated (i.e. during a natural menstrual cycle) IUI results in a higher conception rate compared to no treatment. IUI is consequently often combined with ovarian stimulation using clomifene or gonadotrophins. While this approach is associated with a higher success rate, it also comes with an increased risk of multiple pregnancy. The clinical

pregnancy rate will be increased with more aggressive stimulation regimes, for instance a higher gonadotrophin dose, or allowing women with many mature follicles to undergo the IUI procedure rather than cancel the cycle. In UK practice, triplets are viewed as a major complication and so clinics often cancel the IUI cycle if there are more than two mature follicles. This will accordingly limit the IUI success rate. The IUI success rate per cycle is generally in single figures and the need for patent fallopian tubes, and sufficient sperm, limits its applicability to those who have a chance of natural conception anyway. Many couples are better off moving on to IVF, which has a significantly higher success rate with the benefit of having control over the rate of multiple pregnancy, particularly when elective single embryo transfer is used.

IVF is the most successful treatment for couples with unexplained infertility. Importantly, the success rate is not generally related to the duration of infertility, unlike IUI where couples with more than three years of infertility have a very low pregnancy rate. Consequently, the longer the duration of unexplained infertility, the greater the difference in success rates between IUI and IVF and more appropriate IVF becomes.

Advanced maternal age

As women age, the chance of conception, whether natural or with fertility treatment, reduces. To an extent this can be overcome during IVF treatment by replacing greater numbers of embryos. Currently, in the UK, the HFEA permit a maximum of two embryos to be replaced in women under the age of 40, but three in women older than this. Clearly this carries a risk of triplet pregnancy, though the absolute risk is very low for women approaching their mid-forties. IVF has a success rate in very low single figures for women aged 44–45 years and, for this group and beyond, oocyte donation may be indicated. Preimplantation genetic screening (PGS) during IVF has been suggested as a method of attempting to overcome the increased rate of oocyte aneuploidy, which is the cause of the lower success rate in older women. However, many older women produce insufficient embryos of suitable quality for biopsy and genetic analysis. There is controversy over the extent to which PGS is of benefit in increasing the live birth rate per cycle started (rather than per embryo transfer) when advanced maternal age is the indication.

Male infertility

The most appropriate treatment depends on the degree of semen abnormality and cause, and also the situation with the female partner, for example her age, ovulatory and tubal status.

For men with azoospermia the treatment will depend on the cause. For primary testicular failure (raised serum FSH and low testicular volume), surgical sperm retrieval (SSR) is associated with a 30–50% chance of retrieving sperm. There is an inverse correlation between the FSH level and the likelihood of retrieving sperm with percutaneous needle biopsy. The sperm is usually cryopreserved and used during a subsequent IVF-ICSI cycle, or the SSR can be performed on the day of oocyte collection and used fresh for ICSI. However, this approach risks not having sperm available for insemination and either needing to use donor sperm, or freezing or discarding the uninseminated oocytes.

For men with normal FSH levels and testicular volumes (obstructive azoospermia) the likelihood of retrieving sperm on SSR is 75–95%. It is possible that such men have an epididymal block that is potentially reversible with surgery. Referral to a urologist is required for contrast studies, though very often the site of the obstruction is not found or cannot be repaired. The exception is men who have had a vasectomy. The success rate of vasectomy reversal is related to the length of time since the vasectomy was performed. Successful reanastomosis is less likely beyond seven years. Antisperm antibodies may be present in the ejaculate following reversal which may affect the chance of natural conception. Many men will opt to move straight to SSR followed by IVF-ICSI rather than attempt vasectomy reversal. If the man has diabetes, a neurological condition or has had prostate surgery, then it is possible he has retrograde ejaculation. A post-ejaculation urine sample is examined for the presence of sperm. Some men with a neurological condition, such as paraplegia, may have erectile failure which responds to electro-ejaculation. Alternatively they may undergo SSR. Other men with erectile failure may respond to a drug such as Viagra.

Men with low levels of FSH and a diagnosis of hypogonadotrophic hypogonadism, possibly due to Kallman's syndrome, are offered induction of spermatogenesis using gonadotrophins. Different regimes exist, though most utilize two or three subcutaneous injections each week of hCG and FSH. The response

rate is high, though this can take many months and may not be complete.

Donor sperm treatment is used by many couples with an infertility diagnosis of azoospermia. This may be because sperm is not found on SSR or the couple does not want to, or cannot (for instance for financial or female factor reasons) undergo the procedure followed by the required IVF-ICSI. Furthermore, men with a translocation or other genetic cause of their male factor infertility may prefer to use donor sperm rather than consider preimplantation genetic diagnosis during IVF. Donor sperm can be used for intrauterine insemination or during IVF.

Men with mild male factor and a partner with patent fallopian tubes can consider IUI, though success rates are limited just as they are for couples with unexplained infertility. At least 5 million motile sperm per ml are needed after sperm washing. IVF may be more appropriate and cost-effective. As the severity of male factor increases, IVF is indicated along with ICSI as the severity worsens further.

There continues to be debate over the benefits of zinc, vitamins and other supplements to improve male fertility. Some studies suggest improved semen quality and/or reduced DNA fragmentation. Furthermore, a recent Cochrane review has suggested improved live birth rates in the partners of men taking anti-oxidants. However, the optimal dose and duration of anti-oxidants is unclear. Further study is warranted.

Single women or same-sex female couples

Single women or lesbian couples may be referred to the fertility clinic for treatment. A full fertility history, as described in the previous chapter, is taken to try to determine underlying pathology which may affect the success of donor sperm treatment. If tubal disease is thought to be unlikely, then donor insemination treatment is commenced. The pregnancy rates are < 15% per cycle depending on female age. Ovarian stimulation is used if there is ovulatory dysfunction. If pregnancy does not result after three treatment cycles, or if tubal or pelvic abnormality is thought possible, then an HSG or laparoscopy and dye is performed. Up to six donor insemination cycles are appropriate, though the majority of successes occur during cycles one to three. If insemination treatment is unsuccessful, or if there is tubal damage, then IVF using donor sperm is indicated.

Conclusion

Completion of appropriate and timely investigations allows the physician to discuss and offer suitable fertility treatments with the couple. These may range from expectant management up to IVF-ICSI with PGD. It is vital to be realistic with the chance of success and to explain the risks and any financial costs of treatment to allow the couple to make an informed decision.

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Chapter 18

Social aspects of using reproductive technology

Renate Barber and Alison Shaw

Social aspects of fertility and infertility

The milestones of marriage, parenthood and grandparenthood are taken for granted during a normal life span in most human societies. Couples are expected to have children and grandparents expect to have grandchildren. It is often a personal and social tragedy if, after some years of marriage, there are no offspring; childlessness may detract from a person's self-respect and social standing, besides inviting questions from other members of the family and community. Moreover, these questions are very often aimed at the woman more than at the man. Although infertility, as one of the main reasons for a lack of children, is understood by the medical profession to arise from either the man or the woman, in many societies the woman was, and continues to be, blamed for a couple's childlessness. In fact, in predominantly male-oriented societies the very concept of male infertility has often been totally alien and the phenomenon may still scarcely be recognized, with the blame for childlessness tending to be laid at the woman's door. In societies that permit a man to have more than one wife, it is relatively easy for a woman to observe that her husband does not have children with his other wives either, but the responsibility for childlessness would never be attached to the husband; a wife in this situation might clandestinely contrive to get pregnant by another man (such as a relative). It is only modern investigative technologies including that of the sperm count that have enabled the onus of some infertility cases to be placed firmly on the male.

Concern about fertility is universal and ancient. It is reflected in rituals that in many societies originally centred on the agricultural year and are concerned with producing good yields and fertile earth and fauna [1]. Pagan and Christian rituals, for example, include Easter, which marks the start of sowing and growing of crops, autumn harvest festivals to give thanks for the crops and Christmas, the winter solstice, to mark the ending of the dark season. Almost universally in human history, human fertility has been desired and valued, while barrenness has been feared and disapproved, for powerful socioeconomic and cultural reasons. In subsistence societies, children are valuable economically, by providing labour power. Among some African cattle herders, for example, there is a delicate balance between the size of the herds and the size of the families, because there must be enough people to look after the animals, but there can only be a limited number of people who can live off the herd. In societies lacking systems of social security and insurance for old age and sickness, children are also the only guarantee that the elderly will be taken care of. Where there is high infant mortality, it is thus good policy to have many children to ensure that some will survive into adulthood.

Children also represent perhaps the only visible means of continuity in many traditional societies, by perpetuating a line of inheritance and by enabling property to be inherited by descendants. Societies vary worldwide in whether they are patriarchal (investing power and authority in males), matriarchal or allow both males and females to hold positions of power and influence. They also vary as to whether they calculate inheritance patrilineally (in the male line), matrilineally (in the female line) or along both lines of descent (bilaterally). However, a majority of societies worldwide are or tend to be patriarchal and patrilineal. In such societies, having sons is especially important. For example, in certain forms of male ancestor

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

worship, only men may officiate at the rituals and the rituals themselves must be performed by the man's sons. As a result, there is often considerable pressure not just to have children but to have sons rather than daughters.

The social pressure on a couple, and usually particularly on a woman, to have children is particularly acute in societies in which reproduction is understood to be a female domain and where a traditional division of labour by gender provides the central principles around which family life and the wider society is organized. If, after a few years of marriage or even sooner, a new wife does not become pregnant, she may be vulnerable socially and emotionally, for she is failing to fulfil her expected role as a wife and mother. Moreover, her childlessness may provide grounds for divorce or for the husband to take a second wife, or for the husband's relatives to insist that he takes a second wife. In many other parts of the world today, including at least until recently in the West, the structure of society and the subordinate position of women within it has been derived to a large extent from the fact that women bore and reared children. Indeed, some feminist scholars have argued that men were envious of women's creative power as well as in awe of it, and thus branded women of childbearing age as impure and polluting, particularly during menstruation. This distinction may be marked by rituals of gender separation and isolation, and in consequence of this women have been barred from sacred offices such as the priesthood and other positions of power and authority [2].

The relegation of women to the domestic sphere, which is congruent with the exclusion of ritual impurity brought on by menstruation and childbirth (most Christian churches still 'church' a woman six weeks after childbirth), was largely responsible for the low position of women in many societies and for their general disempowerment. This is underlined by the fact that older, postmenopausal women tended to enter public life to a greater degree, could become highly influential and, in some situations, could adopt socially male roles. Mothers of important men are admired, though their status really derives from that of their sons. The very fact of being the bearer of sons and heirs gave women of reproductive age and capacity a certain position of power, in that their non-cooperation would be a serious threat to a man, but the corollary of this is that women who failed in the childbearing role were at a serious disadvantage and subject to stigma and abuse.

The preoccupation with virginity, which has been and often still is so pronounced in the Judeo/Christian/Muslim world, is due to the need within patriarchal and patrilineal societies to be quite sure that the begetter of children is the mother's husband. Hence women must be under the guardianship of their fathers, brothers, husbands or sons. With a sedentary lifestyle and ownership of property, it became vital that heirs should be of the blood line, and so women had to be closely guarded, their sexuality controlled. The danger of a woman bearing a child conceived from outside the lineage drops away with the menopause and cessation of childbearing. In these societies, women who do not fulfil their biological roles by being barren, or by only having daughters and no sons, are permanently disadvantaged and discriminated against – throughout their lives – unless their childlessness is part of an allotted role such as that of vestal virgin, sworn virgin or nun. The status of a young woman may remain negligible, but the role of 'mother' is honoured. Young as well as older mothers have status by virtue of having given birth, and there is ambiguity as to whether childless women, even those in important positions, are not held in lower esteem than women who are mothers (or mothers as well as having a profession). Under certain circumstances, mothers of many children enjoy greater veneration than young starlets may garner, for example. In the Soviet Union, for instance, 'heroines of the soviet union' were mothers of 12 or more children. Moreover, in patrilineal societies, brides are strangers in their husband's kin group and they will only become full members of their marital family when they have borne children, and thereby become well known and respected for their knowledge and experience.

Techniques and technologies enabling the separation of sexual activity from procreation have had very far-reaching consequences for women's roles which are still working themselves through in the modern world. By being able to control family size, women may choose to relegate childbearing to a relatively brief span in the life cycle. In principle this means that age is no longer a defining factor in role allocation and that motherhood ceases to be a way of life but becomes instead a stage in life. Indeed, gender roles in the modern world are less rigidly determined by such facts as ascribed sex at birth, which no longer must dictate whether we are men or women, mothers or fathers or even whether we must marry people of opposite gender. In relation to women's roles, the

development of reliable contraception was the prerequisite for the emancipation of women. Control of fertility has offered women the freedom to make choices and to move out of the private into the public domain.

Other developments in reproductive technology have increased the potential for change in gender and social roles while also being of service to traditional as well as modern societies. Artificial means of reproduction have the potential for modifying traditional structures of kinship, by allowing people to have children by other means than the simple biological facts of begetting and conception. Artificial insemination by donor, for example, brings non-kin, known or unknown, into the family and surrogacy confounds birth mothers and rearing mothers. Since people are biological organisms and the social persona is closely related to the physical/biological nature of human beings, any rigid separation of the social and the biological (as in the debate over nature versus nurture) seems mistaken, since the two domains are interdependent and bound up with one another. The discipline of medical anthropology acknowledges this fundamental insight, while the medical professions recognize that sociocultural elements are important factors in treatment.

Yet the management of reproduction by man-made techniques raises complex social issues as well as offering means to alleviate childlessness. Mostly people tend to live by traditional values, so although using new reproductive technologies offers a means of escaping the stigma of infertility, the resort to medical technological intervention is also a burden and must often be kept secret. Hence it is essential that medical staff in infertility clinics exercise empathy and discretion and cater to the need for anonymity and concealment. The use of donors in IVF can be controversial as to whether the identity of the donor is revealed or not. In the United Kingdom it is now law that the donor be known in case a person wishes to trace their descent. However, there are implications of suddenly being held to account for paternity many years later that now deter would-be donors from offering their services. Yet in other countries patients may prefer to have known donors, preferably members of their own family, a practice that could be illegal under other circumstances. If for instance the donor is the husband's brother, there are potential complications for the existing family structure, so it is better to keep such an arrangement secret. Consequently arrangements for semen donation may have to be clandestine. Hence clinics must not be too conspicuously located while still being accessible to clients. It is also therefore likely that patients will come from further afield rather than making arrangements in the locality where they are known. This could have implications for support during the often demanding period of IVF and similar treatments.

Comparable considerations apply to egg donation and surrogate motherhood. In practice it means that there is confounding of mother and aunt (if the donation is between sisters as is not uncommon) or of birth mother and social mother. It can be embarrassing to need to have recourse to infertility treatment, especially if it is the husband who is infertile, as this threatens his masculine self-image. Investigations that implicate the man rather than the woman may need especially skilled staff to communicate such findings. Providing semen should be allowed to take place discreetly and privately rather than in an environment with other patients. In societies where sex is private, restricted and shameful, medical practices in infertility clinics are sure to be anxiety provoking and may infringe the rules of normal behaviour. But such is the pressure to have offspring that people will put up with the indignities and problems and pain pertaining to infertility treatment. Moreover the desire / need to produce a son may result in repeated treatments.

One may speculate whether not having sons can be equated socially, in some contexts, to being infertile, so that people pursue numerous pregnancies or practice abortion and infanticide of girls. The easy and relatively cheap availability of amniocentesis in India has greatly increased these practices. Apart from such general considerations, most people have a strong biological urge to have children and particularly children who are genetically their own, which means that sperm and egg donations are means of last resort. Personal dilemmas can be very acute. Before presenting at an infertility clinic, couples have to confront the fact of their inability to procreate. Usually there has to be discussion and agreement between husband and wife to seek such treatment. Moreover, infertility treatment is expensive: couples must consider what they can afford and whether government assistance is available. In Austria, for instance, couples must be aged under 40 for the wife and under 50 for the husband to be entitled to 70% of the costs for two treatment cycles.

Treatment for women is uncomfortable and time consuming and of uncertain outcome. It may even be

dangerous. Doctors are aware of the social and emotional pressures and so may downplay the health risks, realizing that the life of a childless woman is so unpleasant and stigmatized as to have other considerations pale in comparison. Thus, cultural expectations are added to the personal unhappiness of not having a baby. Even where dynastic pressures are less, the continuing enquiries about expected children can be demoralizing, and parents and grandparents can become dreaded influences. Thus the potential benefits for new reproductive technologies are great, provided careful thought is given to how they are presented and what their implications might be.

Global reproductive technologies in local contexts

The uses of in vitro fertilization (IVF) and other technologies of assisted reproduction are now increasingly global, with IVF clinics offering services to childless couples in a wide range of non-Western settings as well as in the Western world where these technologies were first produced. In the Muslim Middle East, for instance, there is an expansive and expanding private IVF industry. In these widely different social contexts, local patterns and understandings of kinship, family, marriage and religion shape the uses of new reproductive technologies, and the use of these technologies can, in turn, have a transformative effect on local social and cultural practices. Some cultural patterns and social trends are discernible in couples' uses of IVF across different contexts, as described below. Nonetheless, it is important not to prejudge any individual or couple's social attitudes or religious beliefs in relation to the use of these technologies, to avoid social, cultural and religious stereotyping, and to recognize that patients may make choices that may counter cultural norms or dominant trends.

Couples' access to and choice of techniques of assisted reproduction may be influenced by prevailing local patterns of kinship and marriage, including ideas about the mechanisms of biological inheritance and the causes of infertility, repeated miscarriage, infant death or childhood illness, as well as the local religious or moral stances of relatives and friends. Legal and religious rulings on the permissibility of the uses of IVF and associated techniques (such as preimplantation genetic diagnosis and selective termination of pregnancy) provide a backdrop against which couples negotiate their use of new reproductive technology, sometimes significantly constraining choice and sometimes offering novel options and opportunities. Wealth, social class and the ability to travel may also significantly facilitate or restrict the use of these technologies. State-funded health systems may allow controlled or negotiated access to the use of new reproductive technologies, while privately funded health systems may permit more open access while simultaneously excluding the poor.

Kinship, inheritance and identity

Theories about social inheritance vary cross-culturally: they may be patrilineal, matrilineal or bilateral, and they may or may not prioritize perceived genetic or 'blood' ties. In South and South East Asia, as well as in the Muslim Middle East, systems of patrilineal kinship prioritize blood links or lineage through men, such that, in Pakistan and in India, for example, a person's kinship or caste identity is considered to be inherited from the father, and being sure of a child's biological or genetic paternity may therefore be a central concern. Patrilineal kinship systems also tend to accord women a more passive role in conception, often utilizing the analogy of (male) 'seed' and (female) 'soil', in which the father makes the prime generative contribution to a child [3]. A contrasting example is that of Jewish identity, which, in Israel and elsewhere, is perceived mainly as inherited from the mother as a consequence of gestation and birth [4].

Cultural theories concerning kinship, identity and inheritance may in some cases provide models from which people draw when understanding genetic inheritance, perceiving genetic material too as being inherited either, or primarily, through men, or through women, or bilaterally. For example, people in patrilineal kinship systems may associate genetic inheritance with patrilateral kin (relatives on the father's side) more strongly than with their matrilateral kin. A couple may thus consider that blood is 'stronger' on the father's side than on the mother's side. It does not necessarily follow from this that a genetic or inherited problem in a child will necessarily be attributed to the father: on the contrary, couples in this situation, or their wider families, may have alternative explanations for the problem, attributing it instead to the wife's behaviour during pregnancy, or to environmental or spiritual causes. Even so, ideas about patrilineal inheritance of genetic substance may

play a part in influencing decisions about managing genetic risk. For example, in some British families where marriages are conventionally arranged within the family and where, in addition, there is an identified inherited genetic condition in a child, parents may think that arranging the marriage of an unaffected child to a relative on the mother's side of the family will mean there is less or no risk of the condition arising in a child of that marriage than if the marriage is arranged with a relative on the father's side, where the blood is 'stronger' [5].

This association of stronger genetic risk with inheritance through men is at odds with the principles of Mendelian genetic theory. Genetic theory recognizes that DNA underpins relationships between biological kin and that each parent makes an equal genetic contribution, via the gametes, to a child, with a child receiving 50% of his or her DNA from each parent (the mother and the father), 25% from each grandparent, and sharing 50% of his or her DNA with each genetic sibling. It follows then that for patients who may understand biological inheritance to be primarily patrilineal, there is the potential for familial genetic risk to be overlooked among matrilineal kin, where, according to the principles of Mendelian genetics, it is equally present.

Eliciting couples' ideas about inheritance may therefore be clinically relevant in discussions of IVF and gamete donation for couples whose unsuccessful childbearing has been attributed to a recessive genetic condition in a fetus or child. A recessive diagnosis means that both parents are 'obligate' carriers of the condition, recessive conditions being caused by inheriting a mutation in the same gene from each parent. Such couples have a 25% risk of having an affected child with each conception. After repeated unsuccessful pregnancies, in the form of repeated miscarriages or infant deaths or births of affected children, such couples may be offered preimplantation genetic diagnosis (PGD) or gamete donation to manage their genetic risk and ensure they have an unaffected child. These options may be particularly appropriate if prenatal genetic diagnosis and selective termination of pregnancy is unacceptable for personal or religious reasons. In the case of gamete donation where there is risk of a recessive condition, there are several clinically significant ways in which couples' understandings of inheritance may influence their donor preferences. A couple may not initially appreciate that both partners (the man and the woman) are carriers of the recessive condition, a fact that can be important where donor sperm or eggs are being considered for IVF. Some couples in this situation favour egg (or sperm) donation from a relative such as the woman's sister (or the husband's brother), rather than anonymous donation, because of concerns to maintain similarity of 'blood' and the integrity of a family identity or patrilineage. However, from the clinical viewpoint this choice of donor carries a significant genetic risk because the woman's genetic sister (and, equally, the man's genetic brother) has a 50:50 chance of also being a carrier. The couple may also consider that blood is 'stronger' on the father's side, and so a donor gamete from a relative on the mother's side of the family may be associated with no, or lower, genetic risk, compared with a donor on the father's side [3].

Where unsuccessful childbearing has been attributed to a recessive condition, for which both parents are obligate carriers, there is a theoretical risk that any gamete donor will also be a carrier of the same mutation and that a baby conceived by gamete donation will be affected. This risk will vary with the frequency of the condition in the population and the likelihood of the donor being a consanguineous relative. Levels of consanguinity within a population are cross-culturally variable. Consanguineous marriage, which is usually defined as marriage to a blood relative such as a second cousin or closer, accounts for 55% of marriages in parts of North Africa, the Middle East, Turkey and South Asia as well as among recent migrants from these parts of the world to Europe, North America and Australia. It confers an elevated risk of mostly very rare recessive disorders in children because of the greater chance of both partners inheriting a mutation in the same gene from a common ancestor. Carrier tests are gradually becoming available for an increasing number of these conditions, and thus may be used to ascertain the genetic status of potential donors for couples at risk of particular genetic conditions [5].

Legal and religious negotiations of IVF use

The practical and moral negotiations regarding IVF use have taken some strikingly divergent forms in different historical, legal and religious contexts around the world. In Israel, debates by Jewish rabbis have resulted in an intriguing mix of restriction and permissiveness in relation to the use of gamete donation and surrogacy. Since, as noted, Jewishness is perceived

to be passed on through the mother, anonymous sperm donation is permitted. In fact, non-Jewish rather than Jewish sperm may be preferred in order to reduce two further risks that are associated with sperm donation from a Jewish man: these are the risk of perceived adultery between a Jewish man and a married Jewish woman, and the risk of perceived incest occurring where donors are otherwise anonymous and the population is small. Thus, the religious logic promotes the reproduction of Jews with non-Jewish genetic material, and does not privilege genes over other constructions of relatedness and identity. By a rather similar logic, single non-Jewish women are preferred as surrogates because this avoids the implications of adultery between a Jewish man and a Jewish woman, besides reducing the chance of incest occurring unknowingly between Jewish persons. Further, the Jewish state is explicitly pronatalist in encouraging Israeli Jewish women to reproduce and in subsidizing the unlimited use of IVF up to the birth of two live children. Rabbis have been generally permissive regarding the use of anonymous Jewish donor sperm by unmarried Jewish women and Jewish lesbian mothers. As a result, Israel is relatively permissive regarding the use of donor gametes, surrogate motherhood and single and lesbian motherhood [4].

In the nations of the Muslim Middle East, where marriage and producing children are very highly valued, there is a rapidly expanding private IVF industry catering to the needs of childless couples. However, across this region, the use of new reproductive technologies has followed a path that reflects the far-reaching influence of Islamic religious opinions (*fatwa*s) concerning the religiously appropriate practices of assisted reproduction. In addition, there has been an intriguing and significant divergence between Sunni and Shi'a religious opinion regarding third party gamete donation.

Sunni Muslims comprise the majority (80–90%) of Muslims globally, and IVF was first used in the 1980s in the Sunni-majority countries of Egypt, Saudi Arabia and Jordan. An authoritative *fatwa* (religious opinion) from Al Azhar University in Egypt in the 1980s continues to be the dominant Sunni Islamic opinion on the use of IVF. This opinion permits artificial insemination with the husband's semen. It also permits the IVF of an egg from a married woman with her husband's sperm, and the transfer of the fertilized embryo to the wife's uterus. However, third party gamete donation and gestational surrogacy are strictly prohibited because of the involvement of a third party, which is regarded as equivalent to adultery. In addition, adoption of a child produced by an illegitimate means of assisted reproduction is forbidden. The influence of this opinion is evident in the fact that, throughout the Sunni Muslim world, third party gamete donation is illegal and IVF clinical practice broadly conforms to official Islamic discourse. This means that, in Egypt for example, where the patients at Egypt's private IVF clinics are overwhelmingly from the nation's elite and are therefore able to pay for the very costly treatment, couples requiring gamete donation are turned away. Some of these Sunni Muslim couples then seek third party gamete donation in Europe or elsewhere, in Iran or Lebanon, in accordance with the relative permissiveness of Shi'a religious rulings on the use of donor technologies (see below). Similarly, in Israel, Palestinian Muslims may attend Israeli clinics seeking donor technologies. However, most childless Egyptian couples agree unconditionally with all forms of prohibition on third-party donation, surrogacy and adoption [6, 7].

Shi'a Muslims comprise a minority of Muslims globally, and are located in Iran and in parts of Bahrain, Iraq, Lebanon, Saudi Arabia, Syria and South Asia. Until the late 1990s, Sunni and Shi'a religious authorities were in broad agreement on the prohibition of gamete donation. In the late 1990s, however, a *fatwa* issued by Ayatollah Khamenei in Iran in effect permits the use of gamete donation, providing that Islamic rules about parenting and social inheritance are followed. With sperm donation, the child becomes the adopted child of the infertile father, and inherits only from the genetic father. With egg donation, the recipient mother becomes an adopted mother and the child is entitled to inherit from the egg donor.

In fact, though, Shi'a practices of religious reasoning have resulted in a wide range of Shi'a positions on gamete donation. Moreover, Shi'a Islam permits *mut'a* marriage, a form of temporarily contracted marriage between a married or unmarried Muslim man and an unmarried Muslim woman, which involves a payment to the woman. This has enabled some couples to obtain donor eggs legally, polygyny being legal in Islam. However, a married woman cannot have a *mu'ta* marriage for the purpose of sperm donation, because polyandry is not legal. The acceptability of these methods of third party donation continues to be hotly contested within Shi'a Islam, with some Shi'a

scholars following the dominant Sunni prohibitions on all forms of third party donation [8]. Moreover, most Shi'a and Sunni Muslims oppose third party sperm donation because this form of donation confuses the lines of descent that are important in patrilineal Islamic societies. A child thus produced is like an adopted child, who lacks a connection by 'blood' to his or her adopted father, and cannot inherit from him.

Despite this, donor technologies are being offered to patients in some IVF clinics in Iran and Lebanon, utilizing eggs donated by other IVF patients, relatives and unmarried women who agree to *mut'a* marriages. In at least one Lebanese IVF clinic, the egg donors are young non-Muslim American women who travel to Lebanon for a fee in order to donate their eggs anonymously. In an intriguing twist of political irony, the most likely recipients of these 'American eggs' are conservative Shi'as who are members of or sympathize with Lebanon's Hizbullah party [6]. The other users of donor gametes include not only Lebanese Shi'a couples, but also Lebanese Sunnis, and Sunni Muslims from other parts of the Middle East where the use of gamete donation contravenes the dominant Sunni opinion. Sunni Muslim couples from the Arab Gulf States similarly travel to Iran to make use of donor technologies [6]. These are significant developments that illustrate the role of wealth and the ability to travel in enabling these new forms of assisted reproduction. On the one hand, then, the use of IVF and other technologies of assisted reproduction globally is clearly influenced by local political, cultural and religious context, and on the other hand, the uses of these technologies can have a transformative effect on local moral worlds, enabling, for example, gametes to travel across ethnic, national and religious boundaries.

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Chapter 19

From Pythagoras and Aristotle to Boveri and Edwards: a history of clinical embryology and therapeutic IVF

Jacques Cohen

All truths are easy to understand once they are discovered; the point is to discover them.

Galileo Galilei (1564–1642)

Introduction

In vitro fertilization (IVF) has become a routine medical intervention over the past three decades, resulting in the birth of millions of children and culminating in the awarding of the 2010 Nobel Prize in physiology or medicine to Robert G. Edwards. Yet, just 50 years ago IVF was considered science fiction and not at all an obvious choice for treatment of infertility and subfertility.

As astounding as this relatively quick rise may be, the future of IVF promises to be even more so. Inventor and futurist Ray Kurzweil predicts that our knowledge base will multiply thousands of times faster during the next few decades compared to the entire history of science, technology and philosophy. IVF is certain to see major new changes with further integration of genetics, molecular biology and physics. But to anticipate and help shape future possibilities, the past must be understood. Where did we begin and how did we arrive here?

The history of science and technology is defined as a field of history that examines how humanity's understanding of science and technology has changed over time. This now-accepted academic discipline also includes the study of cultural, economic and political impacts of scientific innovation. IVF is a wonderfully broad discipline that demands both historical reflection and frank discussion of complex and profound issues touching on matters of law, politics, culture and ethics.

The reader is reminded that this text is not written by a science historian. Though intended to be unbiased, the narrative draws not only on written history gleaned from historical documents, but on personal experience as well as numerous conversations with scientists and physicians in the field.

The history of infertility treatment, and IVF in particular, can be told in many different ways. Here the story is told from the perspective of basic science, with emphasis on the final steps that led to the birth of the first IVF baby in the 1970s and tribute made to those responsible for paradigm shifts in philosophy that allowed the new reproductive technologies to take form. Moreover, because no medical intervention is possible without the tools that have been made available in surgery and laboratory practice, this aspect is also covered in some detail, in the hope that future historical reflections on IVF and related technologies will include appropriate reference to this neglected area of science history.

From preformation and epigenesis to the discovery of chromosomes and meiosis

It is evident that humans have long been intrigued by questions surrounding fertilization and procreation. Symbols depicting fertility are at least 35 000 years old, dating from the early Aurignacian period shortly

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

after the earliest representatives of *Homo sapiens* (Cro-Magnon) migrated to Europe (Fig. 19.1). However, it was not until well after the introduction of script writing that such considerations were recorded in Western thought.

The first written record of deliberations on reproduction starts with those by Greek physicians and philosophers who evidently were quite familiar with the concept of generations and embryology. They held the belief that a new organism could not only arise through sexual and asexual reproduction, but also through the process of spontaneous generation, a now obsolete principle described in detail by Aristotle (384–322 BC) (Fig. 19.2). Earlier, Pythagoras [570–c.495 BC] introduced the concept of 'spermism', an erroneous theory asserting that only fathers provide the essential characteristics of offspring while mothers supply only a solid substrate. Two millennia later the doctrines of spermism and spontaneous generation were finally proven to be wrong through experiments and observations of Louis Pasteur (1859) who won a contest called by the French Academy of Sciences (Fig. 19.3). However, it must be mentioned that 200 years earlier, the physician and poet Francesco Redi had already raised serious doubts about spontaneous generation by conducting an elegant set of controlled experiments that showed maggots could not arise in a jar of rotting

Figure 19.1 Fine examples of so-called small Venus figurines made by European representatives of *Homo sapiens* of the Cro-Magnon culture during the Upper Paleolithic era of prehistory (from 40 000 BCE onwards). These figures either represent an early form of pornography or some form of worship of the female secondary sex characteristics such as hips, breasts and vulva, possibly reflecting the need of survival through reproduction. Facial and extremity details are under-represented or absent. Artistic and cultural interpretation may be a reflection of our modern opinion and experience.

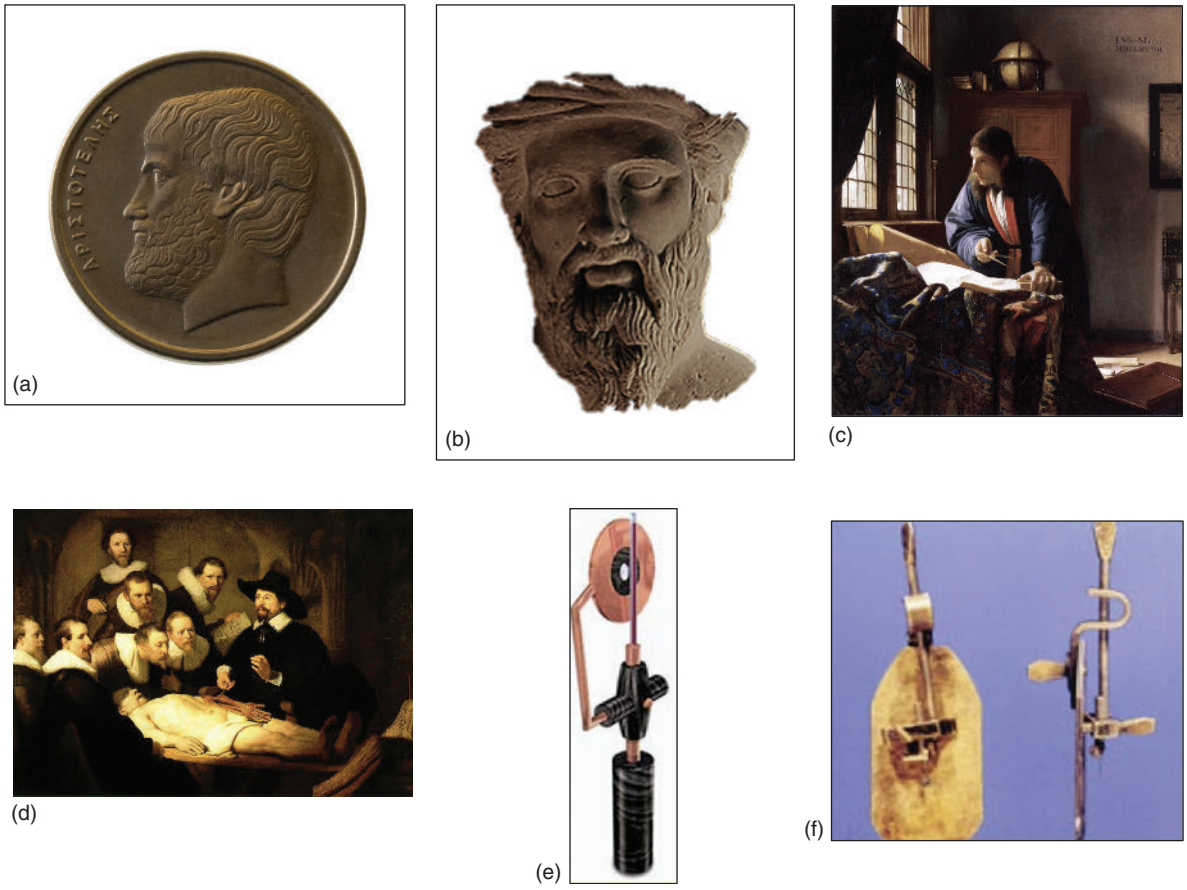

Figure 19.2 (a) A depiction of Aristotle, the great Greek philosopher on a Drachme coin, who first introduced the concept of epigenesis. Reprinted with permission from 123RF. Reprint purchased by author. (b) Rendering of Pythagoras, the Greek mathematician and philosopher, who first formulated spermism. (c) A possible representation of Anton van Leeuwenhoek who first described spermatozoa, in Johannes Vermeer's 'The Geographer'. Vermeer and van Leeuwenhoek knew each other in 17th century Delft, The Netherlands. (d) The figure closest to the pathologist performing the autopsy in Rembrandt's 'The Anatomy Lesson' was mistakenly believed to be the great Dutch biologist Jan Swammerdam. No known portraits of Swammerdam exists. (e) Jan Swammerdam's handheld microscope. (f) Anton van Leeuwenhoek's microscope.

meat covered with gauze. Aristotle's concepts were entirely replaced by germ and cell theories in the nineteenth century, but it took a great deal of convincing before scientists and philosophers accepted that spontaneous generation was simply wrong.

Aristotle described two historically important models of development based on Pythagoras' doctrine known as the theories of 'preformation' and 'epigenesis'. Preformationism held that an embryo or miniature individual already existed in either the mother's egg or the father's semen and began to grow when stimulated; spermism was the first of these models. Aristotle preferred the theory of epigenesis, which assumed that the embryo began as an undifferentiated mass and that new parts were added during development. Aristotle thought that the female parent contributed only unorganized material to the embryo. The male-centric views of the day helped lead him to the conclusion that semen from the male parent provided both the form and the soul. Both Pythagoras and Aristotle were 'spermists'.

Aristotle's theory of epigenetic development dominated the science of embryology until the work of English physician William Harvey (1578–1657), although it took another 200 years to be considered archaic by most scientists. Harvey was inspired by the work of his teacher, Girolamo Fabrici (c.1533–1619). Some science historians consider Fabrici the founder of modern embryology, because of the significance of his embryological thesis: *On the Formed Fetus* and *On*

Figure 19.3 (a) Francesco Redi (1629–1697) was a physician, poet and naturalist who in 1668 elegantly showed that maggots did not form from rotting meat through 'spontaneous generation'. (b) Lazzaro Spallanzani [1729–1799] was an Italian catholic priest and biologist who discovered that reproduction required semen and an ovum. In frogs and dogs he performed artificial insemination, before John Hunter's experiment in humans. (c) Caspar Friedrich Wolff [1733–1794] was one of the first to reject preformationism. His work opened the doors to germ layer theory and fertilization. He discovered the mesonephros. (d) Hermann Fol [1845–1892] was a Swiss zoologist and one of at least three scientists who observed fertilization microscopically for the first time. (e) Louis Pasteur [1822–1895] was a French chemist and microbiologist, best known for developing the first vaccines against rabies and anthrax and the process of pasteurization. He provided clear evidence that spontaneous generation was not an existent reproductive process. (f) Karl Ernst von Baer (1792–1876) was a multi-disciplinary German zoologist born in Estonia. He discovered the ovum in 1826 and the blastocyst later. He also accurately described the germ layer theory of development in the characteristic separation of ectoderm, endoderm and mesoderm.

the Development of the Egg and the Chick. Harvey's *On the Generation of Animals* was not published until 1651 after he completed his ground-breaking, *An Anatomical Study of the Motion of the Heart and of the Blood in Animals* which explained how blood was pumped by the heart throughout the body. Although Harvey had hoped to provide experimental confirmation for Aristotle's theory of epigenesis, his observations proved that many aspects of Aristotle's theory were erroneous, yet Harvey held on to certain core beliefs of epigenesis.

Aristotle believed that the embryo formed by coagulation in the uterus soon after mating. Harvey's experiments in chick and deer eggs persuaded him that generation proceeded by epigenesis, that is, the accumulation of parts over time. Epigenesis or epigenetics is still used in biology, the contemporary sense being aspects of morphogenesis that are not encoded by genes themselves but occur by factors that control the gene activity. Many of Harvey's contemporaries and students rejected Aristotle's epigenesis and turned to the more fundamental theories of preformation.

Naturalists who favoured preformationist theories (preformationism) of generation were inspired by the microscope, probably first introduced in primitive form by two Dutch spectacle makers (Hans and Zacharia

Janssen around 1590) who used their knowledge of lens manufacturing. Based on this primitive compound microscope, Galileo Galilei (1564–1642) added a focusing control. Later, Anton van Leeuwenhoek (1632–1723) refined the curvature of the lenses and his upgraded device could be used to enlarge objects by as much as 260× (Fig. 19.2). Leeuwenhoek was the first to observe bacteria, yeast and blood cells.

Marcello Malpighi (1628–94) and Jan Swammerdam (1637–80), two pioneers of observational microscopy, provided information that seemed to support preformation (Fig. 19.2). Based on Swammerdam's studies of insects and amphibians, naturalists suggested that embryos preexisted within each other and called the forms homunculi or animalcules. This phenomenon was likened to sets of Russian nesting dolls by the developmental biologist and author Pinto-Correia [1] in her outstanding book on preformationism. However, the limitation of this theory was that only one parent could be the biological source of the preformed organism. At the time, philosophers were familiar with the eggs of many species, but when the microscope revealed the apparent existence of 'little animals' in male semen, some naturalists argued that the preformed individuals must be present in the sperm (Fig. 19.4).

Respected scientists of the time, such as Charles Bonnet (1720–93) and Lazzaro Spallanzani (1729–99) supported preformationism (Fig. 19.3). Bonnet's study of parthenogenesis in aphids was regarded as an argument in favour of 'ovist' preformationism. Thus, some naturalists argued that the human race was already present in the ovaries of Eve, while others reported seeing homunculi (tiny humans) inside spermatozoa apparently derived in paternal lineage from the theological figure Adam. Clara Pinto-Correia [1] has argued that the terminology and emphasis on this theory is the result of a more recent historical misrepresentation. The vivid discussions between groups of naturalists and theologians holding these two opposed views would shape the debate on the origins of life for some time to come.

Early cell and germ theories

Some eighteenth-century scientists rejected both the ovist and spermist doctrines. One of the most convincing arguments was raised by Casper Friedrich Wolff

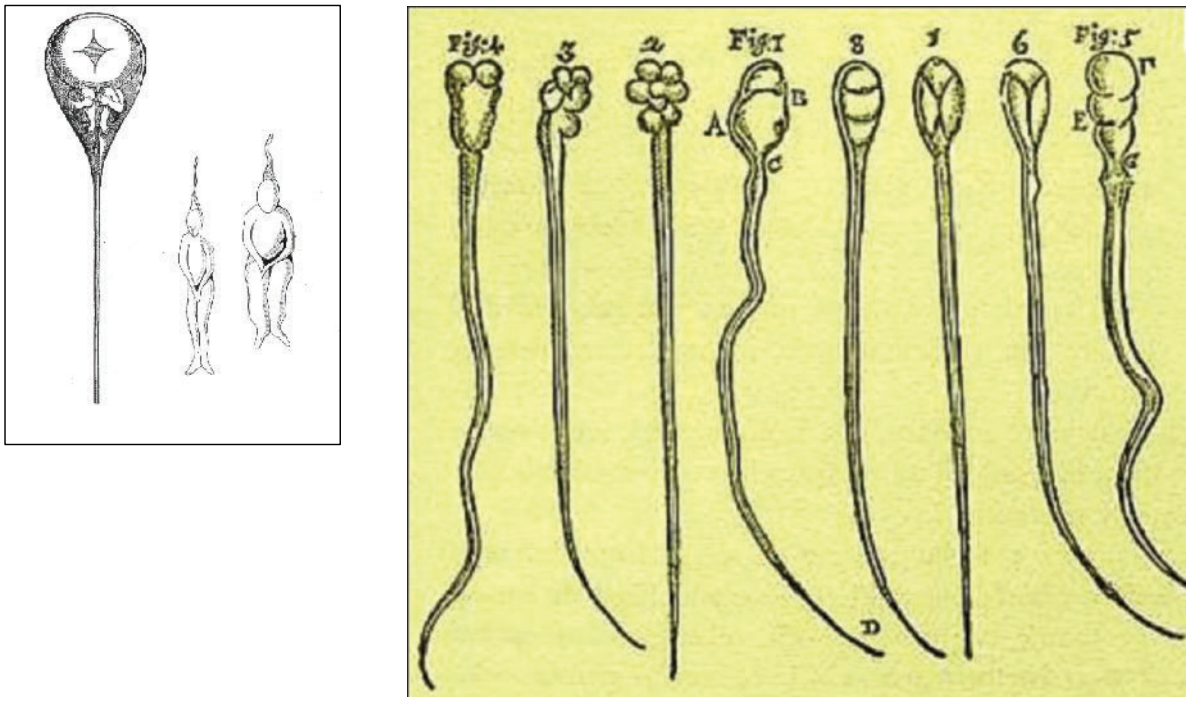

Figure 19.4 Left panel depicts Nicholaas Hartsoeker's homunculi (1695), the presence of a tiny already complete human in the sperm seen using Hartsoeker's primitive microscope. The right panel shows van Leeuwenhoek's sketches of spermatozoa (1677). The latter showed morphologic disparity as well as detailed head features. The differences between the observations of both microscopists may have been due to subjectivity, visualization and artistic interpretation. Hartsoeker never claimed to have actually seen the homunculi, but suggested the representations to support spermist theory. He apparently was present when Leeuwenhoek noticed spermatozoa in semen for the first time.

(1733–94), who published a groundbreaking article, 'Theory of Generation', in 1759. Wolff argued that the organs of the body did not exist at the beginning of gestation, but formed from some originally undifferentiated material through a series of steps. Other naturalists became interested in this attractive model known as natural philosophy. During the nineteenth century, the basis of cell theory was expanded by the discovery (1827) of the mammalian (dog) ovum in Germany by Karl Ernst von Baer (1792–1876) many years after the finding that semen contained millions of individual moving cells called spermatozoa (Leeuwenhoek, approximately 1677; described in Anton von Leeuwenhoek and his perception of spermatozoa by Ruestow).

Historians are not always in agreement about who first actually witnessed the mammalian fertilization process and sperm-egg interaction. Was it Schenk in Vienna, Austria [2] or the Swiss physician and zoologist Hermann Fol [3] (Fig. 19.2)? What is evident is that Schenk was the first to describe the dissolution of cumulus cells in rabbit eggs held in follicular and uterine fluids after exposure to epididymal spermatozoa, thereby clearly establishing the field of experimental embryology.

Figure 19.5. (a) Patrick Steptoe and Robert (Bob) Edwards in 1969 tensely answering questions from reporters during their press conference after the announcement of obtaining proof of fertilization using human gametes in the laboratory. Reprinted with permission of Getty Images. Reprint purchased by author. (b) The first wave of IVF pioneers. From left to right: Pincus, Hamilton and Chang. (c) Walter Sutton, one of the co-pioneers of the chromosome theory of inheritance. (d) Theodor Boveri, the other co-pioneer and the most famous of experimental embryologists of his day. (e) E. B. Wilson who discovered the sex determining chromosomes X and Y, simultaneous with Nettie Stevens (f).

Interestingly, this was reported exactly 100 years before the birth of the first IVF baby in the human [4] (Fig. 19.5).

Oskar Hertwig, a student of the renowned German biologist and artist Ernst Haeckel, described fertilization in the sea urchin two years before Schenk (in 1876) and it seems that these observations led him to emphasize the important role of sperm and egg nuclei during inheritance and the reduction of chromosomes (meiosis) during the generations. Another German biologist and artist, Theodor Boveri, published some of the most significant principles of preimplantation embryology in the late 1880s and early 1890s (Fig. 19.5). Oscar Hertwig before this had already proposed that sperm and egg nuclei fuse during fertilization (fusion is typical in invertebrates studied by Hertwig, but does not occur in mammals).

Boveri studied the maturation of egg cells of *Ascaris megalocephala*, the horse nematode. He observed that as eggs matured, there came a point where chromosome numbers were reduced by half. Boveri was one of the first to see evidence of the process of meiosis. Boveri and Sutton independently advanced the chromosome model of inheritance in 1902 [5] (Fig. 19.5). Boveri performed his studies with sea urchins, in which he found that all the chromosomes had to be present for appropriate embryonic development to occur. Sutton's work with grasshoppers demonstrated that chromosomes are organized in matched pairs of maternal and paternal chromosomes, which detach during meiosis. The Boveri-Sutton chromosome model (the chromosome theory of inheritance) is a fundamental conclusion in genetics. This model identifies chromosomes as the carriers of genetic material. It explains the mechanism essential to the laws of Mendelian inheritance by identifying chromosomes with paired factors as would be required by Mendel's laws. Boveri-Sutton also argues that chromosomes must essentially be linear structures with genes located at specific sites along them. The chromosome as an organelle was discovered at least 60 years earlier by Wilhelm Hofmeister in Germany [6]. Just a few years after Boveri-Sutton, E. B. Wilson and Nettie Stevens independently discovered the chromosomal XY sex-determination system – that males have XY and females have XX sex chromosomes (Fig. 19.5) [7, 8].

Boveri and his partner Marcella Boveri were among the first true experimental embryologists. He was nominated but never received the Nobel Prize before his sudden death in 1912. He chronicled the development of normal sea urchin eggs, but also when the egg was fertilized by two rather than one sperm cells. Boveri deducted that male sperm and female egg nuclei were similar in the amount of transmissible information. They each had a half set (haploid number) of chromosomes. As long as a set of each was present, defined as the diploid number of chromosomes, there was usually normal sea urchin development. Any more or any less and development would proceed abnormally. Mendel's laws were rediscovered in 1900. Boveri recognized the correlation between Mendel's findings and his own cytological evidence of how chromosomes behaved.

The centriole, which is integral to cell division and flanks the spindle, was also discovered by Boveri earlier in 1888 [9]. A pair of centrioles, one aligned perpendicular to the other, are found in the centrosome – the microtubule organizing centre of animal cells (although some centrosomes, like that of the mouse, are acentriolar). Boveri subsequently hypothesized that cancer was caused by errors during cell division. Although scorned at the time, Boveri was later proved to be right. In addition to playing a critical role in mitosis, the centriole apparently also provides structural support. A centriole may have its own unique genetic code, which is distinct from the code of the cell; some scientists now believe that this code allows the centrosome to double and divide with each cell cycle precisely and carry out its various functions in the cell. Boveri correctly argued that only one of the centrioles from the two gametes could survive the fertilization process, the other one being inactivated.

Walter Heape [10] in the UK was the first to successfully transfer a 'segmented ova' (cleaved) embryo from one animal to another. Heape used the characteristics of the Angora rabbit from which the embryos were obtained to describe the offspring after transfer into a Belgian hare. The cohort of siblings was of a mixed nature since the recipient rabbit was mated normally. The embryos were not exposed to laboratory conditions and transfer was done very quickly after washing the embryos from the oviducts. Interestingly, Heape's rabbit experiments were performed either in his laboratory in Cambridge or in Prestwick near Manchester, his family home. Bob Edwards would use a similar venue combination in the 1970s during the first series of human IVF, commuting back and forth between Cambridge and Oldham, a town near Manchester where Steptoe practiced as an NHS consultant. Heape's groundbreaking experiments in rabbits and deer and his suggestion to use the transfer procedures in farm animals in a later

book are described in a concise review by Biggers [10]. Heape's thoughts, to use embryo transfer between two animals, apparently did not translate into the concept of artificial fertilization, at least not explicitly stated as such; however, if his experiments did not lead him to the idea directly, it may have inspired others.

From meiosis to the concept of ectogenesis

The idea of achieving extracorporeal fertilization was probably first introduced by the great British population geneticist, J. B. S. Haldane, who in a book written for a lay audience and published in 1924 [11] described how a process he called 'ectogenesis' would soon create individuals outside of the human body (Fig. 19.6). He predicted that the first birth would occur in 1951, which was only slightly optimistic since the concept would become validated not long thereafter. Haldane's friend Aldous Huxley, an English writer, popularized reproductive technology mixed with provocative descriptions of sexuality some 600 years into the future in his famous novel, *Brave New World* [12] (Fig. 19.6). As has unfortunately become commonplace when the future of science is portrayed, *Brave New World* is a dark prophecy. Huxley only admitted to having copied the concept from Haldane's in vitro conception theory many years after the publication of his book. Now the Heape-Haldane-Huxley concept of alternative forms of procreation was out of the box and the tantalizing possibility that these could soon be available to anyone was on the horizon.

The second paradigm shift occurred with the idea of applying the ectogenesis model to women with tubal disease. This concept was introduced rather plainly in a short editorial in the *New England Journal of Medicine* in 1937 by Dr John Rock, who was a highly regarded ObGyn at Harvard University (Fig. 19.6). At the time, the idea was perceived to be so outrageous that even the author avoided claiming it, and the

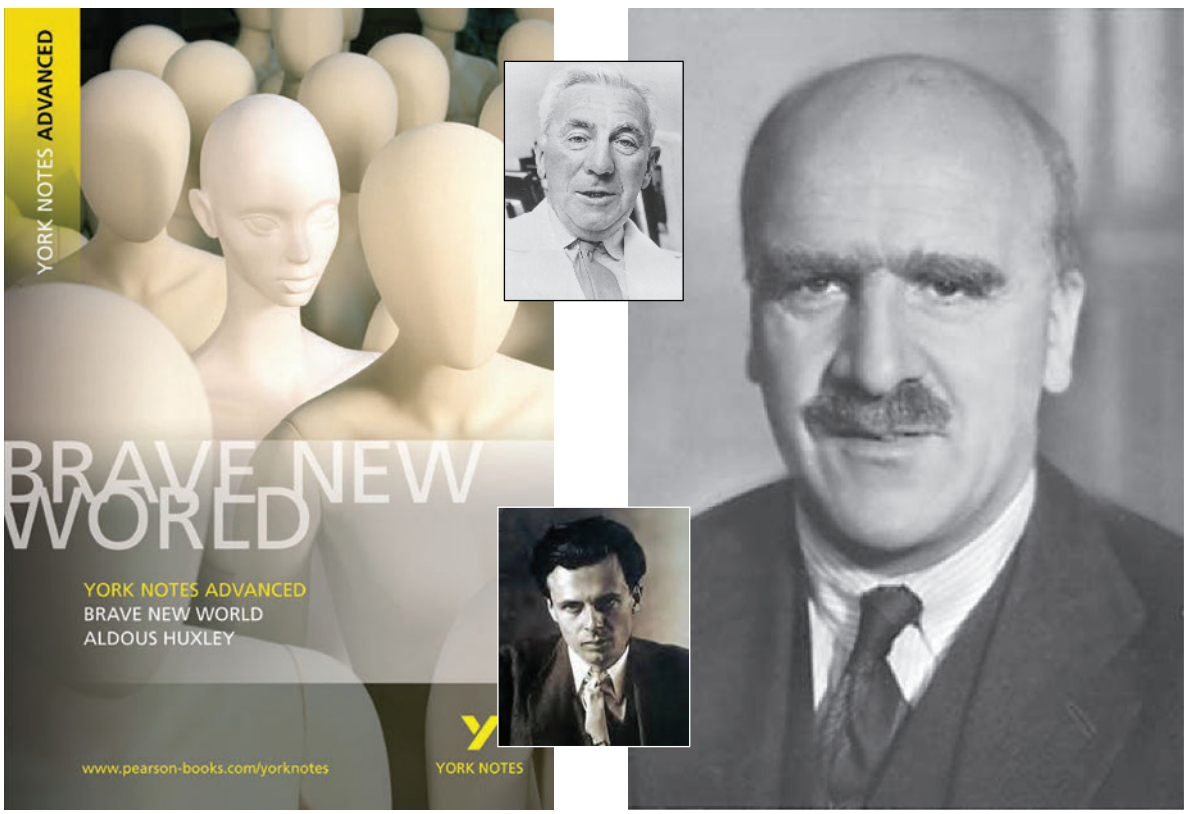

Figure 19.6 A cover of one of the later editions of *Brave New World*, the novel (1932) by Aldous Huxley (lower panel insert) describing a repressed society where anonymous in vitro fertilization and gestation were considered a normal reproductive routine uncoupled from sexual activity. The book was based on J. B. S. Haldane's prophecy of 'ectogenesis' described in 'Daedalus, or, science and the future' (1924). John Rock (upper panel insert), a famous Harvard ObGyn suggested to use ectogenesis for cases of tubal infertility.

editorial was unsigned. The concept had now matured from being proposed as a futuristic way of general procreation to a specific treatment for women with tubal disease.

Infertility diagnosis and treatment before Louise Brown was more sophisticated than is sometimes believed. Infertility was already an established subspecialty well before World War II. By contrast, andrology is a very new discipline. The success of treatment was sometimes expressed as a function of the duration of infertility. Treatment rarely produced better results than no treatment. There were notable exceptions, for instance, tubal disease treatment using surgical intervention was well established and quite successful. Similarly, certain endocrinological and immunological disorders could be treated occasionally. The advent of sperm transfer, artificial insemination using the semen of a donor, may have occurred as early as 1790 in Scotland (Dr John Hunter). In the early part of the twentieth century, donor insemination was practiced sporadically until the 1950s when the procedure was first described in medical journals. Chris Polge was the first to deep-freeze spermatozoa from any mammalian species in 1949 [13]. Human spermatozoa were first successfully frozen in Iowa (USA) a few years later, by Jerome Sherman, who also established the world's first sperm bank in 1960.

Meanwhile, scientists would complete the first steps of ectogenesis in the laboratory, planning fertilization experiments in vitro in animal models. Although M. C. Chang's work in 1959 [14] is widely regarded as the first proof of IVF in a mammalian model, there were dozens of scientific publications spanning 80 years of research, which paved the way for embryologists (described by Austin in 1961) [15]. Of note are the remarkable early experiments by Onanoff in 1893 using eggs flushed from the uterus. Most experimental embryologists later used tubal eggs. Gregory Pincus, the father of the contraception pill, claimed to have fertilized rabbit eggs before World War II [16]; however the does were inseminated first by a buck and the eggs were flushed quickly from the fallopian tubes, after which they were washed vigorously to remove spermatozoa. In the 1950s, when in vitro inseminations were more commonplace, it became obvious that spermatozoa could interact with the zona pellucida shortly after insemination and that excess spermatozoa could not be easily removed by washing. Other observations published by Pincus, such as the presence of two polar bodies after activation (disputed by Chang) and a very short interval of only 12 hours between observing the germinal vesicle and the first polar body appearing in the human (disputed by Edwards), were also reasons to perhaps consider the prewar work in a different light.

John Rock and Miriam Menkin at Harvard would collect hundreds of immature ovarian eggs from patients and attempt to fertilize them with modest results [17]. In the 1950s, Thibault in France and Chang in the United States carried the field forward by confirming fertilization in vitro and obtaining offspring in the rabbit following transfer of the embryos [18; 14]. However, many of the intricate details of the IVF process were still basically unknown. For instance, it was believed that spermatozoa had to mature in the uterus first. By the time Bob Edwards became interested in treating tubal infertility by IVF in 1963, a few others had also attempted to fertilize human eggs in vitro, although fertilization was not positively proven in any of those cases. A number of important questions needed to be resolved first: (1) what was a suitable culture fluid or medium? (2) what was the best way of culturing the specimens? (3) how could immature eggs be matured in vitro? (4) how could mature rather than immature eggs be obtained routinely? (5) how should spermatozoa be prepared? (6) how could more than one egg be recruited? (7) how could ovulation be timed accurately? (8) at what stage should embryos be returned to the uterus? (9) how and where should embryos be transferred? Although some of these questions were being addressed by experimental embryologists working with animal models, each species had its own specific requirements. The human was very different not only because the women were older and suffered from infertility, but because oocytes were obtained from and embryos were returned to the same individual rather than the egg donor and embryo recipient being two different individuals as is routine in animal work. The concepts of clinical IVF and PGD were accurately described in 11 key points published in Edwards' remarkable paper in the *Lancet* [19]. This paper was recently reviewed by one of his first PhD students, Martin Johnson [20].

The culture medium and culture system

For several years after the success of IVF in the rabbit, as Chang [21] describes, 'it was felt that unless living young could be obtained after transplanting such

fertilized eggs into recipient rabbits, successful fertilization in vitro could not be held to be proven, since such eggs could be abnormally fertilized or may not be fertilized at all'. This final confirmation was obtained by Chang in 1959 [14] (Fig. 19.5). He incubated newly ovulated eggs with capacitated sperm for 4 hours, then cultured the eggs in 50% rabbit serum for another 18 hours before transferring the eggs into recipient females. These experiments resulted in the first live births following IVF and embryo transfer in mammals.

It was more than a decade after the discovery of in vivo capacitation in the rabbit that sperm capacitation in vitro was first achieved in the hamster [22]. After another few years, IVF was achieved in the mouse [23], opening a tremendous avenue for research into early mammalian development since the period from egg to blastocyst could now be artificially controlled in at least one species. For experimental embryology in mammals to move forward, a reliable embryo culture method was imperative so that the fertilized eggs could be maintained in vitro through the cleavage stages. Earlier, Hammond [24] had discovered that mouse embryos collected at the 8-cell stage, but not the 2-cell stage, could be cultured to the blastocyst stage in a physiological saline solution that was supplemented with hen's egg yolk and white. Although the inability to culture 2-cell embryos was and remained for some time a formidable challenge, Hammond's discovery was a significant one and set the stage for abandonment of biological fluids as culture media for embryos. In 1956, Whitten [25] replaced Hammond's medium with a modification of Krebs-Ringer bicarbonate solution. He supplemented the latter with glucose, antibiotics and bovine serum albumin (BSA) and showed that it could support development of 2-cell and 8-cell mouse embryos to the blastocyst stage. Two years later, McLaren and Biggers [26] obtained normal young following transfer of blastocysts grown in Whitten's medium, proving that viable blastocysts could be produced in vitro.

At this juncture, it was the inability to obtain large numbers of eggs at a time (and in a controlled manner) that hampered research efforts. According to Edwards [27], the dogma of the time dictated that ovaries of adult females would not respond to gonadotrophic hormones. However, Fowler and Edwards [28] successfully challenged this dogma. They followed the work of Gates [29], who had artificially induced ovulation in pre-pubertal mice using a regimen that included injection of pregnant mare serum followed two days later by serum from a pregnant woman. Fowler and Edwards [28] used the same method and induced superovulation and pregnancy in mature mice. Later, it was shown that superovulation could be achieved in the human using pituitary gonadotrophins [30].

But another hurdle had to be overcome before production, fertilization, culture and transfer of mammalian eggs would become routine practice. A reliable and efficient embryo culture system had to be devised. In 1963, Brinster introduced culture of eggs and embryos in small drops of culture medium under a layer of paraffin oil. With only minor modifications, this 'micro-drop' method using a nineteenth-century invention called the Petri dish, has become the most widely used and successful system for culture of mammalian embryos in vitro today. It would be difficult to think about human embryos growing in the laboratory without contemplating their artificial world and the Petri dish that is temporarily their home. The Petri dish is used in more than 99.9% of ART procedures. By inference, embryologists may have used over 100 million of them to date. In spite of that, the dish has changed little since its inception in the latter part of the nineteenth century. There have been few secondary changes to adapt the original plain design of the dish to areas of specialized use such as cell tissue culture (e.g. the square four-well dish) and microbiology. The same can be said about the adaptations made to the Petri dish after its introduction to preclinical embryo research in the 1940s and 1950s. There have been few such alterations and usually these have been unremarkable, such as place markings for droplets or identification numbers on the bottom. The dish was developed in the latter part of the nineteenth century, because there was a need in vaccine research to grow microorganisms on a solid substrate rather than in a broth. This used to be the common way of growing bacteria in culture until the famous German scientist and physician Robert Koch (1843–1910), known as the master of germ theory, suggested replacing the liquid phase. This made a huge difference to the field of germ culture and development of vaccines, but the problem was that Dr Koch's assistant had difficulty using glass flasks for this purpose. Koch's assistant was Julius Richard Petri (1852–1921). He decided one day in 1887 to cut off the flask and only use the bottom for pouring the solid media into. The dishes were manufactured in glass, and mammalian embryos were

cultured experimentally using glass dishes well into the 1970s. In the mid-1980s, a sudden increase in the cost of raw material and a better understanding of the injection moulding process allowed most manufacturers to reduce the weight of plastic Petri dishes to the 15–17 gram range. This new thin plastic Petri dish has remained largely unchanged and is now an industry standard.

As mentioned above, one of the most important steps towards contemporary embryo culture was developed by the scientist Ralph Brinster (of sperm stem-cell fame) in 1963, when he successfully cultured mouse eggs to blastocysts. He decided to do away with 'open' culture and protect small amounts of culture medium using a transparent viscous fluid overlaying the media. He used paraffin oil for this purpose. The advantages of this system were huge, although it essentially moved away from Koch's solid substrate approach for which the Petri dish was designed. Oil prevented most microbial infections, allowing fertilization and embryo growth events to take place in less stringent conditions. For example, gametes and embryos could be observed for longer periods since medium evaporation became a problem of the past. The method also allowed the study of minute quantities of metabolites released or absorbed by the cells and later, it facilitated the introduction of micromanipulation methods. Intra-cytoplasmic sperm injection (ICSI) would have been nearly impossible without the use of oil. The high heat capacity of oil also helped to maintain incubator temperature when the dishes were moved around for observation or manipulation. The problems were oil toxicity and batch-to-batch variation. Paraffin oil has now been largely replaced by other oils such as mineral oil. This is a variation on light hydrocarbon oils, a distillate of petroleum. Toxicity has been diminished because certain mineral oils are used for human consumption as a lubricant laxative. However, batch-to-batch variation is still a problem. Brinster's technical marvel was for a long time unappreciated by human IVF specialists, as nearly all early practitioners (particularly in the USA) used either organ culture dishes or small test tubes for culture of human gametes and embryos .

Two decades that changed human IVF

The basic principles of experimental animal embryology and experience gained in that area, including oocyte maturation in vitro [19], were first successfully applied to the human in March 1968: Edwards and Bavister, using a modification of Tyrode's solution devised by Yanagimachi and Chang [22] for hamster IVF, added sperm to nine human eggs and, 11 hours later, recorded the presence of a sperm tail within one egg and the presence of pronuclei in another. This was indisputable evidence of fertilization in vitro in the human [31], but it was only the first step since this medium was not able to support further development. It was already known that seminal plasma was not supportive of fertilization and also spermatozoa had to undergo a process called capacitation first, before they could penetrate the oocyte.

The collaboration between Bob Edwards and Patrick Steptoe, one of the most fruitful collaborations ever undertaken between a scientist and clinician, started in 1968 because Steptoe had been able to introduce laparoscopy successfully after others like Palmer (1944) and Fikentscher and Semm [32] provided the instruments to visualize and manipulate the ovaries.

The first infertile patients were invited to participate in IVF treatment in 1970. Unfortunately for those volunteers, it took over 100 transfer attempts to finally obtain a sustained pregnancy in November 1977. The first pregnancy had been achieved a year earlier in 1976, but it was ectopic and had to be terminated. Wood and Leeton in Australia also reported a biochemical pregnancy in 1975. Other teams in Sweden, Holland, the USA, India and Australia had joined in, but the two pioneers remained the most focused and determined about the work in progress often supported by a third collaborator, nurse Jean Purdy. Purdy played a crucial role in the convergence of experimental embryology and reproductive medicine. She facilitated the transformation of basic research in in vitro fertilization to a meticulous clinical discipline with a foundation in quality control. Jean Purdy is without a doubt, the founding mother of QC in clinical embryology.

Louise Joy Brown was born on 25 July, 1978 and quickly became the most famous baby in the world. Her name is still well recognized worldwide. She represents Edwards and Steptoe's quest for knowledge and making human IVF a reality for infertile couples. After the birth of Louise, a short – and remarkably understated – letter was published in the *Lancet* [33]. Three things stood out in this publication. The first was that the transferred embryo was an 8-cell and not a later stage embryo as was the case during previous transfer attempts. The transfer of blastocysts was

based on the assumption that embryos at earlier stages would be received with physiological hostility, since it was believed that the uterus normally accommodates only morulae and blastocysts. We now know that this is only true in animal models and that the human uterus can tolerate any stage of development around the time of ovulation, even pre-fertilization if sperm and eggs are injected together [34].

The second surprising revelation in the *Lancet* letter was that Lesley Brown's (Louise's mother) diseased fallopian tubes were removed and her ovaries had been relocated into a position of easy accessibility. It escaped no one that this manoeuvre guaranteed that there would be no doubt about the pregnancy having occurred with the IVF embryos and not per chance from the spontaneous fertilization of a wandering egg.

A third extraordinary aspect of the announcement was that the mature egg had been retrieved from a naturally growing follicle rather than from follicles that had been developing under exogenous hormonal stimulation, as had been the case in previous patients. The question that was then posed was whether the natural cycle was requisite to success of IVF. It was the team of Alan Trounson that provided the answer a few years later using gonadotropins and clomiphene citrate [35] successfully. Earlier, another team in Melbourne achieved the first Australian pregnancies [36].

It should be noted that the initial and subsequent successes of IVF occurred against an extraordinarily unfriendly background, without the support of government agencies and under a continuous barrage of criticism. Many ethicists, religious leaders, politicians, lawyers, fellow scientists and physicians were appalled by the idea. Edwards confronted them head on and even described scenarios new to them in order to focus the debate. His defence of IVF never wavered and he has written dozens of scholarly articles about the legal, political and ethical issues surrounding reproductive technologies.

Establishing and expanding the clinical alternative: the 1980s

At the end of 1980, Edwards and Steptoe opened the world's first IVF clinic near Cambridge in an old landhouse called Bourn Hall, which became Bourn Hall Clinic; it had taken the founders some time to establish the facility due to the general lack of interest among financiers. Government funding, both locally and nationally, was quite out of the question after the UK Medical Research Council (MRC) and National Health boards again refused to support IVF; an earlier refusal goes back to 1971, when the MRC declined to fund the emerging field of assisted reproduction (for an excellent review on this topic see Johnson *et al.* [20]). Later in 1983, the MRC would again refuse to grant a broad research application from Edwards and his embryologists. Nevertheless, Bourn Hall Clinic became a legendary place complete with in-patient wards, ethics and visitors' committees, endocrinology, embryology, research laboratories, parlours and a dining hall. Other clinics were opened soon: at the Royal Women's Hospital (Alex Lopata) and Monash University (Carl Wood, John Leeton and Alan Trounson) in Melbourne, Australia with some government support, and in London, UK (Ian Craft) from private funding. At the Eastern Virginia Medical School in Norfolk, Virginia (USA), two famous reproductive gynecologists, Drs Georgianna and Howard Jones, opened the first US-based facility using funds released by the university. Other countries such as India, Austria, France, Holland, Sweden and Spain followed swiftly and established their own clinics. By 1982 a new discipline was in the making, a field some people were referring to for the first time as ART or Assisted Reproductive Technology.

The enthusiasm generated by the success of IVF in Norfolk in 1981, however, did not persuade the US government to lift the moratorium it had placed on all human embryo research a year earlier. In fact, later (in 1995) a law was enacted that prohibits the funding of 'research in which human embryos are destroyed, discarded, or knowingly subjected to risk of injury or death greater than that allowed for research on fetuses in utero' (Dickey-Wicker Amendment, 1995). The US federal government thus does not support clinics or any clinical studies and this sad situation has not changed for over a quarter century.

Although the basis of the technology was now established, many of its aspects were poorly understood. A number of important observations had been made by the first IVF pioneers. They recognized that timing of ovulation and follicular recruitment were complicated processes often limiting a team's ability to plan ahead while many patients became frustrated because of cancellations shortly before egg retrieval. Drugs were needed to recruit follicles at will and to control and time ovarian stimulation. The first such family of drugs were the GnRH agonists. These

drugs down-regulate the secretion of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) resulting in a dramatic decline in estradiol levels. This allowed suppression of endogenous gonadotropin production and the LH surge, and planning for egg retrieval following an injection of human chorionic gonadotropin (hCG) [37].

Another clinical bottleneck during the early days of IVF was the requirement for laparoscopy. Although a magnitude more efficient than laparotomy, laparoscopy had to be performed under general anesthesia in a full operating theatre, and required considerable recovery time. Moreover, when visualization was hindered, ovaries remained inaccessible and dominant follicles unreachable. The search for a faster and more efficient means of oocyte recovery was on. Ultrasonography, though in its infancy, had already been applied to track growing follicles [38]. The question was whether it could be used during egg retrieval to visualize the follicle and its content. After all, the ovaries were positioned near the vaginal wall. Nevertheless, the first aspiration of a follicle using ultrasound was achieved trans-abdominally, a considerably longer route requiring access through the bladder [39]. That same year abdominal ultrasound was combined with vaginal follicle aspiration [40]. The final and determining step was performed by the Swedish team of Hamberger and Wikland in 1985 using a new, narrow vaginal ultrasound probe guiding a needle adjacent to it; this method is still in use 25 years later [41].

In the laboratory, meanwhile, experimental embryologists, veterinary researchers and pathology technicians were retrained as clinical embryologists. Their first task was to safely handle and observe gametes and embryos. Laboratory and equipment maintenance and standardization of methods were other important tasks, as was meticulous recordkeeping. These first clinical embryologists were surprised to notice that human embryos varied considerably [42] not just between patients, but also within cohorts. This variability made evaluation of embryos difficult. Even more frustrating was the fact that morphology and rate of development seemed only loosely correlated with outcome. The search for important characteristics that predict implantation has brought under examination many aspects of gamete and embryo development in culture, and complicated algorithms have been developed (see Chapter 30). However, after 30 years, not a single common morphological marker has been identified that can predict with certainty the future success of an embryo. Even algorithms of multiple morphologic criteria do not reveal implanting ability with accuracy. During the past 15 years, researchers have attempted to correlate clinical outcomes with embryo metrics, but with only mixed success. Certainly one of the major challenges remains the identification of accurate (and affordable) embryo selection methods, a crucial step in further reducing multiple pregnancy and facilitating single embryo transfer [43].

IVF is the first and only general treatment for infertility and sub-fertility; couples with male infertility can now be treated just as successfully as those with female-related infertility. However, this aspect was not generally accepted in the early 1980s. It was feared that spermatozoa from men with male infertility would not be able to penetrate the zona pellucida or that if they did, fetal development could be abnormal. However, when couples with male factor infertility were selected for IVF, many had fertilized eggs, although the fertilization rate was only a fraction of that in other groups of infertile couples [44]. Moreover, many men with severely reduced sperm counts could not be treated, as not enough spermatozoa could be prepared for microdroplet insemination. The notion that micromanipulation could enhance fertilization in male factor cases even further than standard IVF had already been suggested some years back. The first such experiments in some human spare eggs were conducted in Rotterdam in 1979 (Zeilmaker and Cohen, unpublished). The first birth in mice following micromanipulation was achieved by opening the zona pellucida artificially, an approach called zona drilling or dissection [44]. In 1988, human babies were born from a similar mechanical zona dissection as well as injection of spermatozoa into the perivitelline space [45, 46]. Though this improved the prospects for treatment of male factor infertility, fertilization rates were low due to the absence of a quick block to polyspermy on the membrane level. This meant that embryologists could only use very low concentrations of suboptimal spermatozoa. Fertilization rates were improved dramatically with the introduction of ICSI by a team of researchers in Brussels, Belgium [47]. ICSI is now the preferred treatment method for those at risk of reduced or failed fertilization (see Chapter 26).

The most exciting events in science are often marked by the merging of seemingly unrelated disciplines. The field of reproductive science had already

experienced this in the nineteenth century, when the beliefs of both spermists and ovists were shattered by the observation that spermatozoa penetrate the egg and that this is followed by the formation of two pronuclei in the zygote. Those lucky clinicians and scientists practicing IVF in the 1980s witnessed not one but two revolutions. The first groundbreaking shift was the enablement of preserving extra embryos for later use. Cryopreservation of the embryo (and later the egg) allowed clinicians to reduce the number of embryos for transfer. In the human, all stages between the zygote and blastocyst were frozen; however, different cryoprotectants and freezing protocols were required [48–51]. Thawing of embryos later allowed transfer in the natural cycle. Some couples did not have to undergo multiple IVF treatments, since the embryos from one cohort could be enough to establish a multi-sibling family. The effort was well founded in science, since pioneers working with rodents and farm animals had already mastered the technology years earlier [52–54). The past ten years have seen further refining of egg and embryo cryopreservation, the aims being simplification of methodology and increasing egg and embryo survival rates (see Chapter 31).

The other revolution in the 1980s was genetic diagnosis of embryos through blastomere biopsy before transfer [55]. Interestingly, the general concept was already introduced 20 years earlier by Bob Edwards and one of his brilliant PhD students at the time, Richard Gardner [56]. They performed trophectoderm biopsy in the rabbit embryo, applied a sexing technique and transferred sexed embryos to the uterus. More than 20 years later and a few years after development of the polymerase chain reaction (PCR), this elegant experiment would form the basis for a new field called Preimplantation Genetic Diagnosis (PGD) (see Chapter 33).

The evolution of reproductive clinical science

IVF is now considered an industry, a field of its own. More than 2000 clinics specializing in IVF exist worldwide. The largest, in Tokyo, Japan treats more than 15 000 couples a year. A few forward-looking governments support the IVF effort financially. Other governments, such as the ones in Sweden and Belgium, support and guide the practice with smart laws based on clinical data. Many professional organizations have been formed to support the effort, and special university-based training programmes exist for physicians and embryologists subspecializing in IVF. It is estimated that 5 million babies have been born through ART; however, the road to this success has not always been easy. In 1934, Dr Gregory Pincus was a young man in his early thirties when he claimed to have achieved in vitro fertilization in rabbits, just a few years after Haldane's prophecies and Huxley's book. While the discovery made international headlines, he was vilified in the press for his research. The *New York Times* depicted him as Dr Frankenstein, just like others would later describe the work on in vitro fertilization (IVF) by Patrick Steptoe and Robert G. Edwards as a travesty. It must have been disconcerting for scientific mavericks like Pincus and Edwards to be called names for their sound scientific enquiry. Yet, maybe they found solace in the history of science, since many true innovators, Copernicus, Galileo, Darwin and Boveri among them, were frequently disparaged and often unfairly treated during their lifetime.

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Chapter 20

Legal, ethical and regulatory aspects of Assisted Reproductive Technology (ART)

Ingrid Granne and Lorraine Corfield

Legal and ethical issues in assisted reproduction are numerous and complex and cannot be covered in their entirety in this chapter. Our aim is to discuss assisted reproduction in the English legal system in some depth, while differing approaches to regulation by other countries are addressed in less detail. Using clinical examples, a number of difficult ethical questions are raised in order to introduce the reader to some basic moral theories, but more important to provoke thought and discussion.

Assisted reproduction and the law

Internationally there are two main approaches to the regulation of assisted reproduction treatments (ART). First, countries such as the UK, Germany and most of Scandinavia have passed laws covering most aspects of ART. Second, many other countries such as the USA have fewer laws in this area, and the regulation of fertility treatments is overseen by professional bodies. There is much debate in the field of assisted reproduction as to where the balance should lie between professional standard setting and legislation.

Regulation of assisted reproduction may be particularly problematic for several reasons. First, science in this area continues to develop at a rapid pace. Consequently, by the time laws are debated and passed, new techniques may become available that could not have been envisaged when the laws were designed. A second reason that makes this area particularly difficult to regulate is the diversity of opinions regarding how technologies should be applied, an obvious example being therapeutic cloning. In addition, what is culturally and socially acceptable may change over time and views of what is acceptable may be radically different between different countries or communities within the same country.

The human fertilization and embryology authority

The UK was the first country in the world to significantly regulate fertility treatment and embryo research by implementing wide-ranging legislation through the Human Fertilisation and Embryology Act 1990 (HFE Act 1990)[1]. This Act of Parliament also created the Human Fertilisation and Embryology Authority (HFEA) [2]. This is an independent regulator overseeing the use of gametes and embryos in fertility treatment and research within the UK. The HFEA licenses fertility clinics and centres carrying out in vitro fertilization (IVF), other assisted conception procedures and human embryo research. It inspects clinics regularly, ensuring standards are maintained and that clinics comply with the law. In addition, it plays a role in informing patient choice by publishing information on treatments offered by individual clinics along with their success rates.

An important function of the HFEA is to issue guidance known as a Code of Practice [3]. A Code of Practice is a legal document accompanying an Act of Parliament that helps individuals and officials interpret the law. The HFEA Code of Practice is intended to help and encourage licensed fertility centres to understand and comply with their legal requirements. It also provides guidance on how centres are expected to go about meeting those requirements. Clinical embryologists working in the UK will be very familiar with the Code of Practice, as it gives practical guidance on all areas of their work.

The Human Fertilisation and Embryology Act 2008 (HFE Act 2008)

In the 20 years following the 1990 Act, enormous scientific advances (for example stem cell technology,

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

preimplantation genetic screening and research possibilities with ad-mixed embryos) meant that the legislation did not cover many of the developing technologies. In addition, public opinion on many of the complex ethical questions posed by fertility medicine had changed. Over the years individuals had applied to the courts to decide difficult legal issues such as creating 'saviour siblings' who are HLA type-matched to provide donor stem cells for a sick child. Such dilemmas had not been envisaged when the 1990 Act was conceived. A comprehensive explanation of the HFEA 2008 cannot be covered in this chapter. However the main areas that are covered by the Act are reviewed.

Assisted Reproductive Treatments (ARTs) require a licence from the HFEA

The Act states that fertility treatments can only take place in centres that hold a licence for those specific activities. A named individual is the licence holder for a particular clinic. Activities that require a licence are wide ranging (see Fig. 20.1).

Legal responsibilities of assisted reproduction clinics

The Act places many legal requirements on fertility clinics. It does not suffice for a clinic simply to be licenced for the treatments it offers. The Act requires clinics to report all the treatments undertaken, requires that patients are given adequate information regarding treatments and even places a legal requirement on clinics to consider the welfare of any future child and to have strategies in place to reduce the number of multiple pregnancies. Once again, not all of the responsibilities of clinics are detailed here, but the most important are considered below.

Procurement and processing of gametes or embryos
- Any process by which eggs, sperm or embryos are made available, transported or delivered
- Any operation involving the preparation, manipulation or packaging of eggs, sperm and embryos

Storage
- Storage of eggs, sperm or embryos by freezing

Treatments
- Insemination
- In vitro fertilization
- Use of donor gametes or eggs
- Gamete intra-fallopian transfer (GIFT)
- Zygote intra-fallopian transfer (ZIFT)

Techniques
- Pre implantation genetic diagnosis (PGD)
- Pre implantation genetic screening (PGS)
- Assisted hatching
- Zona drilling
- Subzonal insemination (SUZI)

Figure 20.1 Activities requiring a licence under UK law.

The person responsible

The law is clear that licenced activities may only take place under the supervision of a 'person responsible' who is named on the clinic's licence. That person is usually (though not always) a clinician and must have a sufficient understanding of the scientific, medical, legal, social and ethical issues involved. That person is ultimately responsible for ensuring that all licensed activities are conducted with proper regard to the law.

Information and consent

The law is quite specific that clinics must provide individuals with enough information as to the nature, purpose and implications of their treatment. They must have the opportunity for adequate counselling about the implications of their choices and decisions, and they must be aware that they can withdraw consent at any time.

The law requires written informed consent to be obtained from an individual prior to:

- storing gametes or their use in the treatment of others
- creating in vitro embryos, their storage or use in treatment
- using embryos (created with their gametes) for research or training
- using an individual's cells to create embryos for research
- using an individual's cells or gametes to create human admixed embryos
- disclosing information about an individual's treatment, for example to a GP or another clinic

There are specific consent forms published by the HFEA for each of these circumstances. These forms also ask patients to decide in advance what should be done with their stored gametes or embryos in the event of their death or mental incapacity.

Welfare of the child

Clinics are legally obliged to take into consideration the welfare of any child born as a result of licensed treatments. Clinics have refused to treat individuals because of their concerns about the welfare of any

resulting child, although this has on occasion been challenged in the courts. This clause in the Act is an example of how views have changed over time. In the original 1990 HFE Act, the law stated this consideration must include the need of a child for a father. Eighteen years later, as treatment of single women or homosexual couples has become increasingly socially acceptable and commonplace, the law was revised, requiring clinics to consider the need of a child for supportive parenting.

Multiple births

Some of the most significant risks of IVF are those associated with multiple pregnancy. Currently the law allows a maximum of two embryos to be replaced in a cycle unless a woman is aged 40 or over, when a maximum of three embryos may be replaced. The HFE Act 2008 addresses this directly by requiring licenced centres to have a documented strategy to minimize multiple births. In effect this means each clinic must have set criteria that, if met, mean that women should be offered elective single embryo transfer. A log (which can be reviewed by the Authority) must be kept of cases where women had two embryos transferred when they met the clinic's own criteria for single embryo transfer. In such cases the clinic must record the reason for deviating from this policy.

Adverse incidents

Clinics are legally required to inform the HFEA of all adverse incidents or 'near misses' occurring at the clinic. These include events such as ovarian hyperstimulation syndrome (OHSS) requiring hospital admission, loss or damage to gametes or embryos or any case of misidentification of embryos or gametes.

Use of gametes and embryos

Storage of gametes and embryos

The law allows gametes and embryos to be stored for a period not longer than 10 years in most cases. This can be extended in some circumstances (for example if a teenage boy has sperm stored prior to chemotherapy). Valid written consent is required prior to storage and an individual can withdraw their consent at any time. The law is very clear that in the case of stored embryos either partner can withdraw their consent at any time. Therefore, if a couple had been treated together and had stored frozen embryos and the relationship ended, one partner could not use the embryos if the other did not consent and either partner would have the legal right to allow the embryos to perish if they did not wish them to be used.

Embryo testing

The law allows preimplantation genetic diagnosis (PGD) to establish whether an embryo has a genetic, chromosomal or mitochondrial disorder that may affect its capacity to result in a live birth. In addition, PGD is legal to avoid the birth of a child who would develop a serious disability, illness or medical condition. Of course what constitutes a 'serious medical condition' is a matter of much debate [4].

The law expressly forbids screening embryos in order to select the sex of a child for social reasons (such as family balancing); however, PGD may be used to choose a child of a particular sex if the disorder being screened for affects one sex much more commonly than another (as in hemophilia). Clinics have to apply for a PGD licence for each specific condition they wish to screen for, such as cystic fibrosis or Duchenne's muscular dystrophy. Embryos that are shown to be abnormal cannot by law be replaced if there are other non-affected embryos available to replace.

The issue of 'saviour siblings' is a good example of how technology moved forward very rapidly after the first HFE Act was passed in 1990. Requests were made to the English courts to use PGD in order to HLA tissue match embryos. The resulting tissue-matched child when born could then donate cord blood in order to treat a sibling with a life-threatening illness. These controversial cases provoked much media, ethical and religious debate regarding the commodification of human life. The courts initially decided that tissue typing could only take place if PGD was already being carried out to choose an embryo that would not be affected by the same disease (such as avoiding an inherited life-threatening anemia such as beta thalassaemia). This decision was subsequently revised to allow saviour siblings to be created as potential donors for any 'life-threatening' disease in a child. The HFE Act 2008 went even further, allowing IVF with PGD tissue to create a sibling who could after birth provide cord stem cells, bone marrow or other tissues not only for siblings with life-threatening conditions but also to address serious illnesses.

Gamete donation and surrogacy

The law regulates recruitment, screening, payment and anonymity of egg and sperm donors. Sperm

donors cannot usually be over the age of 46 and in most circumstances egg donors must be under 36. They must be screened for a family history of inherited disorders. Screening including karyotyping, testing for infectious diseases such as HIV and Hepatitis B and C along with sexually transmitted infections must be undertaken prior to accepting an individual as a donor. Since donor sperm can be successfully frozen, a six-month quarantine period must be observed before the sperm is used in treatment. Donors must be informed that any child born as the result of donated eggs, sperm or embryos has the legal right to identifying information about the genetic donor once they reach the age of 18. Donors are allowed to be reimbursed reasonable expenses for their donations but cannot be paid for their donation.

Although the HFEA does not regulate surrogacy directly, it does regulate all donated gametes. Therefore patients providing gametes in a surrogacy arrangement (for example the husband of a couple in a surrogacy arrangement may provide sperm as a 'donor' for intrauterine insemination of the surrogate) must be registered as donors and screened as donors.

Research and training

The law specifically regulates the use of embryos in research and training. First, no human embryos can be kept or used for the purposes of research for more than 14 days or after the primitive streak has appeared, if this is earlier than 14 days. A research licence must be applied for in order to undertake any research on embryos. This licence must be obtained for each specific research project and patients must give written consent for their embryos to be used for a specific project. Patients must also give written consent for embryos to be used for training (for example, intracytoplasmic sperm injection – ICSI).

Legal definitions of parents

When a woman is treated using her husband's sperm, or embryos created with his sperm, her husband is automatically the legal father of any child born. If a married woman is treated using donor sperm or embryos, once again her husband is treated as the legal father (unless the husband did not consent to the treatment, for example they were separated and he was unaware of her treatment). Parliament had to consider the changing nature of the family in the twenty-first century when drawing up the HFE Act 2008. Given that civil partnership between homosexual couples is legal in the UK, this was reflected in the Act, giving both parties in civil partnership the status of legal parents of any child born using donor sperm. In cases where couples are not married, they must both consent to the male partner being treated as a legal parent, and where two women are being treated using donor sperm, they must both consent to the second female being treated as a legal parent of any resulting child.

International regulation of assisted reproductive treatments

In the confines of this chapter it is not possible to detail extensively how each country is regulated. Instead, we will look at some of the underlying influences on regulation and why different countries may choose to regulate as they do.

Influences on regulation

Culture and society

Certainly the prevailing liberal or conservative nature of a country may directly influence how governments choose to regulate reproductive treatments. In addition, many societies have strong religious influences on government policy.

Religious beliefs are an overriding influence in the regulation of reproductive treatments in the Arab world, where little legislation exists but the practice of ART tends to abide by religious laws. An example of this is that the use of donor gametes or the use of surrogates is not acceptable. This is because there is a religious and cultural import placed on genetic lineage. This can also be seen with regard to the use of cryopreserved embryos. In the UK, a woman may use cryopreserved embryos, even after the death of her partner, as long as her partner gave consent for them to be used by her before his death. Indeed, the HFEA consent forms specifically ask for individuals to decide and consent to what should be done with cryopreserved embryos should death or mental incapacity happen to one or the other of the parties. In contrast, in Arab countries this would be unacceptable, as genetic lineage can only be assured if both members of the couple are alive.

In stark contrast, libertarian views in the USA mean that only a few aspects of fertility treatment, such as certification of embryology laboratories, are regulated by federal law. Commercial surrogacy and gamete donation are multi-million-dollar businesses,

sex selection is legal and there is no legal limit on the number of embryos that can be transferred. Regulation is largely in the hands of professional bodies such as the American Society of Reproductive Medicine who publish good practice guidance.

The only area of assisted reproduction that has significant federal law is embryo and stem cell research. This is almost certainly a reflection of the influence of Christian groups and their objection to the inevitable destruction of embryos. Until the change of administration in 2008 there was a federal ban on state funding for embryo and stem cell research. This has been lifted recently.

Societal beliefs about fertility

Some societies actively encourage their population to increase in size, and this may be reflected in how a state regulates fertility treatments. One example of this is the state of Israel, where there is a higher rate of IVF use than any other country. The government actively encourages population growth, and this is reflected in the state funding of IVF which is almost uniquely generous, funding cycles until an individual has two children. Unlike many countries, marital status is not an issue.

Historical influences

Germany's embryo protection laws prohibit the creation of more than three embryos, and all those created must be replaced. The embryo in German law comes into being after syngamy: thus it is legal to fertilize more than three oocytes, but all 2 pronuclei (2PN) embryos must be frozen and subsequently transferred. This means that no selection for embryo quality can be made and screenings such as PGD or PGS are illegal. It has often been noted that there are unintended undesirable consequences of such restrictions. Although 2PN embryos have a low implantation rate compared to selected day 3 or day 5 embryos, there is still a significant risk of triplet pregnancy and the associated fetal morbidity and mortality. Triplet pregnancy is also considered an indication for selective fetal termination to reduce the pregnancy to a twin or singleton. This relatively prohibitive legislation is often explained as a reaction to fascism and the spectre of eugenics.

Reactive legislation

As we have previously seen, the rapid pace of scientific development in reproduction has often meant that regulation is not put into place in particular countries until problems arise. In 2008, the Indian government passed the Assisted Reproduction Regulation Act. This was largely in response to the particular issues of sex selection of embryos and an increasing trade in commercial surrogacy. The gender imbalance in India is well documented. Termination of female fetuses is illegal although widespread and it became clear that gender selection in IVF was an increasing problem. In addition, foreigners were commonly commissioning Indian surrogates and paying far less for such arrangements than is typical in the west. There was no regulation of this practice and there were numerous difficulties with subsequent international adoptions. The Indian Assisted Reproduction Regulation Act makes sex selection illegal and regulates surrogacy arrangements. In addition it requires licencing of fertility clinics and regulates embryo research.

As has been shown, large differences exist between different countries in their approach to the regulation of assisted reproduction. The growing industry in 'reproductive tourism' is likely to continue to increase as people are prepared to travel and pay for treatments that are not legal, or are more readily available, in countries other than their own. As technology advances, the difficulties of regulating reproduction are likely to become ever more acute.

Assisted reproduction and ethics

> Procreative liberty has a firm moral basis in the importance that reproductive decisions have for individuals. Such decisions are among the most important that an individual will make in her lifetime. Having or not having offspring will determine central aspects of her personal identity and definition of self . . . if the genetic characteristics of expected offspring would affect that decision, it would appear that prospective parents should be free to use genetic information in making those decisions. [5]

Reproductive autonomy (also referred to as procreative liberty: the freedom to make choices about reproduction) is a highly important individual and societal value. However, this needs to be balanced with other moral priorities and with societal interests:

> Because those [reproductive] decisions help shape the nature of the society in which others will live, there is some case for collective societal decision making . . . the greater the harm would be to another as a result of respecting a particular reproductive choice, the weaker is the overall moral case provided by self-determination for respecting that choice. [6]

Ultimately, the impact on society will depend in part on the moral opinions of the individuals in that society as well as of the society as a whole. This risk of harm to society through providing or withholding access to certain reproductive technologies is a fascinating area, but the discussion here is limited to relevant moral arguments at a more fundamental level.

The spectrum of ethical debate in this area is vast and this chapter cannot provide a comprehensive analysis. The basics of some well-established moral theories are presented where appropriate in order to provide a starting framework for considering each debate. These should not be taken as the only way to address each argument but merely as a way of initiating the non-philosophically trained into the world of medical ethics.

Case 1

A reputable IVF clinic approaches its regulatory body with a request to allow the clinic to offer free IVF to women who would not otherwise be able to afford the treatment, provided the women donated any unused embryos for implantation into other women.

On the face of it, this is an attempt by the clinic to address the inequality in access to IVF by a mutually convenient arrangement. Justice is an ethical priority in healthcare, particularly for those who subscribe to the four principles theory of medical ethics (autonomy, beneficence, non-maleficence and justice, as described by Beauchamp and Childress [7]). If this system is used, the topic under consideration is assessed ethically according to each of these four principles, and the choice that is in accordance with all, or most, of the principles is seen as the ethically 'correct' choice.

The request by the clinic raises concerns about coercion: it is unavoidable that many of those who cannot afford unconditional IVF may consciously or subconsciously feel compelled to donate any spare embryos. As we saw earlier, free will is central to reproductive ethics. However, one could argue that it is still the choice of the individual as to whether to accept the offer (after all, how many choices in our lives are really unconditional?). One well-recognized moral theory, that of consequentialism, requires that the action that confers the greatest good for all should be undertaken. This allows the negative effect of coercion provided that it is 'outweighed' by the positive effect of more equal access to IVF and a beneficial effect on the couples concerned.

No discussion of reproductive ethics can avoid the difficult question of the moral status of the embryo. Views on this are often strongly held and may be polarized. For those who believe that the embryo has full moral status, the approach offered by this clinic may be partly welcomed, as it will reduce embryo destruction. Interestingly, it is worth considering whether the status of an embryo created in vitro differs, as it has no chance of becoming a baby without assistance [8].

Case 2

Judith (66) has wanted children all her life, but she only met the right man a year ago. Dan (63) hasn't had children either and would love them. Judith and Dan are fit, healthy, well-educated and wealthy.

This raises one of the most cited arguments in favour of assisted reproduction: the right to have children. The United Nations Universal Declaration of Human Rights, Article 16 states that 'men and women of full age, without limits due to race, nationality or religion, have the right to found a family' [9]. The practical ethical question here is whether this is confined to a *negative* right, a right simply not to be prevented from founding a family, or whether it extends to a *positive* right, a right to be helped by others, specifically the state, in the founding of a family. In other words, is there a duty to provide assistance for reproduction if needed? Deontology is a moral theory in which individuals have absolute or relative duties to others. These can be negative duties, such as the duty not to kill or not to lie, or positive duties, such as a duty to help those in need. Such an approach would certainly demand a negative duty not to prevent an individual(s) having a family without technological assistance, but whether one has a duty to provide this assistance would depend on possible conflicting duties to others, to society and to the embryo (if one considers the embryo to demand duties from others).

The effect on society and others then comes into play. Putting the moral status of the embryo to one side, the morality of providing or withholding assistance is likely to require balancing the pros and cons of each eventuality, whether that is done from a consequentialist, deontological, four principles or other ethical approach. However, individuals are generally free in society to do as they wish, with positive assistance if required (although the individual may have to pay for such assistance), unless the behaviour involved harms others. It follows then that if the technology exists to help an individual reproduce and make a genetic choice about the embryos re-implanted, withholding it must be justifiable.

An oft-cited concern in assisted reproduction is the welfare of the child. However, if reproductive assistance is given to Judith and Dan the resulting child can only be born with Judith and Dan as parents: that particular child cannot be born to other younger parents. On one level therefore, the welfare of the child argument only holds weight if the life of the child would be so terrible that he or she would be better off never having existed as that child can only have the one life or no life. However, ethical arguments have little practical value if considered in isolation: if positive assistance is to be offered by professionals and by society, it may be reasonable to limit this assistance to parent(s) who can provide a reasonable quality of life (if it is even possible to define what that would be) to the resulting child. However, this then counters the moral values of equality and justice (see above).

Case 3

Susan is a carrier of Duchenne Muscular Dystrophy (DMD). She and Mark want IVF so they can select an embryo that neither has the disease nor is a carrier. They already have one son who has DMD. Neither Susan nor Mark is infertile.

The following list of questions concerning embryo selection in such scenarios will provide a starting point for considering some of the central ethical debates in such scenarios. It is not possible to discuss any in detail but the aim is to provide food for thought.

- A child with DMD is likely to be wheelchair bound by his early teens but may well have several years at least of worthwhile life. To what extent does the severity of the disease affect the permissibility of embryo selection?
- Does it make a difference if the genetic abnormality has variable penetrance, such as with BRCA1, a 'breast cancer gene'?
- Does the age of onset make a difference: is the moral argument for selecting against embryos carrying a gene for a late-onset disorder such as Huntington's disease weaker as there will be many years of presumably worthwhile life?
- Who should make these decisions? Should it be the parents, professionals, a board of ethicists or those running the country concerned?
- Is there a real difference between selecting against disease and choosing the 'best' embryo?
- Does the added burden to a mother of knowing she has passed on an X-linked disorder make any difference to the decision (compared with a spontaneous disorder such as Down's syndrome)?
- Does society have a duty to encourage selecting out embryos with genetic disorders for the sake of future generations? What are the ethical concerns if society insisted on selecting against some disorders?
- What does selecting against disability, such as DMD, state about societal views and acceptance of existing people with disabilities? Does it give a message that those with disabilities are less valued than those without? If the existing son discovers that his parents have undergone IVF specifically to have a second child without DMD (that is, unlike him), what effect will this have on the son and on the family relationships?

The above three cases, discussion and list of questions are designed to stimulate thought rather than provide moral answers. As difficult as it may be, it is vital to address these topics as the effect of assisted reproduction decisions on individuals is immense, and the potential impact on current and future society huge. No person working in the field of embryology should ignore the moral conundrums his or her work produces. For further discussion regarding the ethical and legal implications of ART, please refer to Chapter 36.

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Chapter 21

Quality management in assisted reproduction

Janet Currie and Jo Craig

Introduction

What is a Quality Management System (QMS)?

A quality management system (QMS) is a means of ensuring that quality of the product or service you are providing is consistently of the highest standard and meets specific legal requirements. It is also a way to ensure continuous improvement by concentrating on the daily working practices of the clinic. In order to do this all areas of the clinic must be monitored for performance and efficiency.

Why is a QMS important?

It is often perceived that the success of a clinic is mainly dependent on the IVF laboratory. However the patients' experience begins long before their gametes reach the laboratory, so it is important that the QMS system addresses all areas of the clinic. A well-run QMS will help ensure that the patients receive the best possible level of care, from their first phone call to the clinic to the end of their treatment cycle. Patients will always be disappointed if their treatment fails, but if their experience was one of unreturned phone calls and being kept waiting for appointments, they are unlikely to return for further treatment even if their actual medical care was of the highest quality.

The QMS is important within the IVF laboratory because, in order to increase success rates, it is first essential to be able to demonstrate that clinical procedures are consistent and reproducible. This requires competent staff, properly maintained equipment and appropriate consumables. If these are not in place, results will be unreliable and any plans for improvement cannot be monitored effectively.

Regulatory requirements

It is mandatory for all UK centres offering licenced fertility treatments to have an effective quality management system in place. Since 2007, the HFEA (Human Fertilization and Embryology Authority) has included this as a condition of granting a licence to practice and is related to Article 16 of the EU Tissue and Cells Directive of 2006. The HFEA lays out its requirements for a QMS system in its Code of Practice; however, the specifics of how this is implemented are left to the individual clinic [1]. A broader approach to quality management can be found in the standards available from the International Organization for Standardization.

ISO – International Organization for Standardization

ISO is one of the world's most important developers of standards and represents an international consensus on what constitutes best practice. There are currently more than 17 500 International Standards which range from the specification for the size of credit cards to the constituent materials of concrete. Although the standards are voluntary, they may be referred to by laws and regulations in member countries.

ISO 9001: 2008

These are the International Standards relating to quality management and can be applied regardless of what the company or organization does. They provide a systematic approach to managing processes within the organization by specifying what is required in the QMS but not how the requirements are met. An organization can be inspected against this standard by an independent quality system certification body

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

in order to obtain certification of conformity. In addition to the clinic benefiting from a well-run QMS, obtaining ISO certification can prove a useful marketing tool in today's business-oriented climate.

The standards relate to the following areas, which will be discussed in more detail:

- Infrastructure and work environment
- Organizational setup and responsibilities
- Communication
- Documentation
- Management of resources
- Control of non-conformances
- Measurement, analysis and continual improvement
- Validation of equipment and processes
- Measurement and monitoring of equipment
- Identification and traceability

The processes implemented as part of the QMS should be incorporated as part of the daily working procedures of the clinic, so that each happens automatically rather than being seen as specialist areas set aside from normal practice. All staff should be involved, which is crucial if the QMS is to be successful. It is important to note that many of the processes required are not new to those who work in assisted reproduction, but as a QMS requires that specific standards are met for each process this may necessitate changes to working practice.

In this chapter we will discuss what is involved in a quality management system and some of the ways in which it can be implemented in an assisted reproduction clinic.

Infrastructure and work environment

The efficiency of any service is affected by the environment in which the work takes place. The space allocated for work cannot usually be altered but the internal environment can. A cluttered or badly organized office, clinic or laboratory will influence the way staff members carry out their tasks and will not only slow processes down but also increase the risk of error. There are also health and safety issues here relating to the ease of maintaining high standards of hygiene and the storage of equipment. Simple examples are fire extinguishers which must be safely secured to the wall and easily accessible, and not storing heavy items of equipment on high shelving.

Critical in the organization of any ART clinic is the consideration of issues linked to patient confidentiality. This includes not only the storage of confidential material but also the environment where patients are seen, as they must not be able to read or hear identifying information about others. Storage of medical notes should be well organized and each set effectively tracked so that they can be located easily. The introduction of electronic notes, already in place in some clinics, significantly increases work efficiency in this area.

In the design of any new facility, consideration of adequately sized areas for efficient working practices in laboratory, clinical and all patient areas is crucial. Other important considerations include the ability to easily move equipment in and out of the building, as evidenced by siting the laboratory on the ground floor to enable safe, easy delivery of liquid nitrogen supplies.

Organizational setup and responsibilities

The staffing structure of the whole clinic must be defined in an organizational plan with clear lines of reporting responsibilities for each member of staff. It must also include the responsibility for the QMS for each of the teams. Every clinic must have a quality manager who has direct access to the senior managers and is involved in senior management meetings where decisions are made. Each staff member must have a job description that clearly outlines their job and to whom they report.

It is helpful if the responsibility for specific roles or tasks is allocated to a named member of staff, with a second person acting as backup when required. This is particularly useful for maintaining standards in each room or area to ensure that each is kept tidy and well stocked. This allocation of responsibility includes designation of legally required persons such as a complaints officer and health and safety officer. This latter role covers all areas and includes considerations from the safe disposal of sharps to evacuation in the event of a fire.

Communication

Good collaboration and communication both within and between the various teams working in the clinic are crucial if efficiency and success are to be achieved. In addition to team meetings, regular meetings of the whole clinic ensure that staff are updated on clinic activities and are given an opportunity to provide feedback or present ideas they may have for improvements. In a busy clinic this can be an area of weakness,

as it may be difficult for staff to find the time to attend meetings, which can then significantly impact the effectiveness of the quality system. Time spent listening to problems and identifying areas that are not running as efficiently as they should is time well spent and must always be found. Minutes should be taken at all meetings, and these should be available as a record of decisions made and also as a way to update any staff who were not in attendance. Other effective methods of communication within the clinic include bulletin boards and electronic communication such as e-mail.

Communication with patients is another crucial area, and all staff must be providing the same information. It is therefore essential that all the members of each team are kept up to date with any proposed changes or improvements. Patients are more likely to express dissatisfaction with issues relating to communication with the clinic than with their actual medical care.

Documentation and records

The specific quality requirements of the QMS relate not only to the documentation that is required but also to the presentation of all documentation and to how it is organized and controlled. It is important to remember that documents can be presented in hard copy, as well as via computer, in pictorial or in video form. Documentation includes internally generated documents such as SOPs (Standard Operating Procedures), patient information sheets, data sheets or worksheets, and externally generated documents such as equipment instruction manuals, training and reference manuals or advice sheets.

Documentation required for the QMS includes a quality manual, a quality policy and quality objectives.

Quality manual

The quality manual is useful for all new employees, as well as for visitors who want to know more about the clinic. This document briefly describes the history of the clinic and introduces the senior management team and their responsibilities. It describes how the clinic operates with regard to different areas such as communication, documentation, and health and safety. It outlines the services provided and the treatments carried out and also provides links to all critical SOPs. It explains how the centre complies with the requirements of the regulatory bodies, such as how standards of documentation are met, mechanisms in place for feedback and the control of monitoring and measuring processes.

Quality policy

All ART clinics must provide and display a quality policy in their workplace. This document summarizes the overall aims of the clinic relating to the standard of performance to be achieved and quality of care provided to all who are affected by the work carried out. It should be simple, concise and should reflect the whole ethos of the clinic.

Quality objectives

These relate to the quality policy and the achievement of the promises made there. These will be further discussed later in the chapter in the section on 'Measurement, analysis and continual improvement'.

Standard Operating Procedure (SOP)

An SOP is a protocol that describes step by step how a specific procedure should be carried out. Each should be easy to follow and should state where, when, by whom and with which materials and equipment each activity must be performed. They are a very important part of the QMS as they are a means to ensure procedures are being carried out as agreed and also will facilitate regular audit. Staff must perform any task, whether in the laboratory, clinic or offices, according to the instructions in the SOP; this will lead to overall conformity and reproducible results. All SOPs should be easily accessible and must be reviewed annually. Discipline SOPs also include those pertaining to team management and equipment monitoring, as well as all procedures carried out by that team.

Document control

The organization and control of documents is a key component of the QMS because it affects all areas and greatly impacts the efficient running of the whole system. Documents should be logically organized and easily located by staff. There must only be one version of any document in circulation at any one time, and this must be the most up to date version. Every document should have a standardized header and footer, with the version number and the date of the most recent review usually documented in the footer.

A document review plan should be created and adhered to in order to ensure current practice is

reflected in the SOPs and the information given to patients is evidence-based and up to date. Similarly, consent forms must always reflect current legislative requirements. The frequency of review of the various document groups should be decided by the senior management team. Critical documents such as SOPs, consent forms, information sheets and standard letters should be reviewed annually. The responsibility for the control of documentation, including all amendments and approvals, must be allocated to specific staff, and any changes must go through this person(s). This is crucial to prevent unauthorized changes or the introduction of non-approved documentation. The document control process should be facilitated by the setup of access privileges on the IT computer system, with most staff able to read or print documents, but not granted the ability to make changes or save any that have not been authorized. Once any document has been amended, procedures must be in place to ensure old versions are replaced with the latest one in all hard copy locations. If a central server is not in place feeding a network of computer terminals, then individual computers must all be simultaneously updated. This latter option is far from ideal and where possible central servers should be in place.

Records

Records must be maintained on every aspect of work undertaken. This obviously includes the results of any treatment carried out, including who did each task with the date and details of each. It also refers to the equipment used, and linked to this are the servicing dates and maintenance records together with the monitoring and measuring charts. Patient records must be maintained according to all regulatory standards set, including confidentiality clauses. Specific quality records relating to the effective implementation of the QMS, such as corrective/preventive actions, incidents, audits and quality indicators must also be kept up to date.

Management of resources

Resources include staff, suppliers and all services provided to the clinic.

Staff management

Staff members arguably are an organization's most important resource, and the QMS requires documented evidence that their needs are being met. This refers to clear job descriptions and responsibilities, together with how their continuous professional development is facilitated, including dates of courses, appraisals and training. It also requires that staff have been signed off as competent before they are permitted to perform any tasks unsupervised. There should be clear evidence of induction and training programmes for all new staff members. For trained staff, competency checks should be carried out at regular intervals. Other assessments of proficiency will be made by means of regular performance figures and operator audits.

Suppliers

The efficiency of the clinic is intimately linked with selected suppliers reliably providing goods and services that meet the clinic's needs. Third party agreements must be set up with any company providing goods that have an impact on gametes or embryos. It is a good idea, however, to have agreements with all suppliers. The terms of the business and standards required can be clearly stated and companies must sign to agree to the terms and conditions. It is a requirement of the QMS that the services provided are regularly reviewed to ensure that the contract with any company is still adequate and to reconsider the contract if the agreed standards have not been met.

Control of non-conformances

Problems will arise in any working environment, and a non-conformance can be defined as a failure to meet a specified requirement. An important requirement of the QMS is that a very clear procedure must be in place if anything happens that could adversely affect the quality of patient care or the efficient running of the clinic. This will also include any near misses. Any problems arising must be recognized and all staff should know how to report them so that they can be discussed and steps taken to address the problem. Corrective or preventive action must then be put in place and disseminated to staff. It is very important that a date for review is set to assess the effectiveness of the action. Only when the agreed action has been fully implemented and proved to be effective can the non-conformance be signed off and closed. It is crucial that all these steps are fully documented so that any problem and its stages of subsequent reconciliation are easily tracked.

Non-conformances may be identified from the following sources:

- Adverse incidents
- Audits
- Complaints

Corrective or preventive action can also be applied to other situations where the need for change has been identified, such as patient satisfaction questionnaires or staff suggestions.

Measurement, analysis and continual improvement

In order to show continual improvement there must first be systems to measure the current performance of the clinic. All aspects of the QMS should be covered, including staff performance, patient care and treatment success rates. Then, after plans for improvement have been implemented, they can be assessed for effectiveness.

Methods to monitor performance and promote improvement include:

- Audits (including operator audits)
- Customer satisfaction questionnaires
- Quality objectives
- Quality indicators and key performance indicators

Audit

It is essential that all staff carrying out a specific activity are doing it in the same way and correctly following the SOP. Therefore checks must be carried out to document proof of conformity, and this is usually done by auditing.

Horizontal audits look in detail at a single SOP (e.g. semen preparation) and the auditor will normally watch several members of staff performing the same procedure. In contrast, a vertical or process audit will follow through a longer process which may cover several different SOPs and involve different members of staff at each stage. For example, a vertical audit looking at patients booking for IVF treatment may follow how each patient is handled by the administrative person taking their call, the nurse processing their paperwork, the doctor prescribing the drugs and the finance office billing for the cycle.

Both types of audit are important and as many staff as possible should be trained to carry them out. It is preferable that staff do not audit their own areas. Familiarity with a specific procedure can affect objectivity, while detachment and a fresh pair of eyes are more likely to identify practice that is not in conformance. However, audits must be carried out in such a way that they are not seen as 'finger pointing exercises'. They should be perceived by staff as non-threatening evaluations of procedures, done for reassurance as well as to identify areas requiring improvement. Patient confidentiality must be maintained and individual feedback should always be given to staff involved and their managers. Presentation of results to the rest of the clinic should be carried out without specifically identifying any staff member.

The main focus for audits should be the critical areas where any procedural non-conformity could make a significant difference to the outcome of treatment. Other areas to audit are those identified as not performing efficiently, often discovered as a result of an incident or detected by staff members. Most audits will be internal audits, but external audits are carried out when the regulators inspect the clinic and the processes involved. It can also be useful for staff from other clinics to carry out external audits, providing valuable inter-centre comparisons with the ultimate aim of identifying best practice and overall improvement.

Operator audit

Operator audits should be carried out at regular intervals to compare the performance of staff in critical areas. This can only be done where the outcome is measurable, and care should be taken to remove any inherent bias. For example, the pregnancy rates for different staff performing embryo transfers should be assessed at regular intervals. However, the data examined should be for patients within the same age range and similar embryo quality, as these are factors known to have a significant effect on the outcome. In the laboratory, the performance of ICSI practitioners is one useful area to monitor. Operator audits allow any underperforming staff member to be identified and their practice further analyzed. In some circumstances additional training may be indicated to improve performance. Operator audits can also identify those staff with very good results, and examination of their practice could lead to increased success rates for other practitioners. Care should be taken to make staff as comfortable as possible with this process; the aim is to support staff improvement, not to demoralize underachievers.

Other areas where internal quality control is important are semen analysis and embryo grading. For these procedures the important factor is consistency between individuals performing the same procedure. External quality control is useful to compare the clinic's performance in these areas with other clinics. For some processes, such as semen analysis, there are national schemes such as UK NEQAS (National External Quality Assessment Service) in which the clinic can participate.

Customer satisfaction questionnaires

It is easy to assume that the service provided meets the patients' requirements; however, evidence is needed to demonstrate whether or not this is the case. Clinics must provide documentation to show that the satisfaction levels in different areas have been assessed. Provision for feedback from patients and referring doctors is an important part of quality management and allows the clinic to continually improve the services they offer and the satisfaction levels of the groups concerned.

Questionnaires are a very useful tool because they can be modified for use in any area and are often anonymously completed. This allows patients who may not like to be identified to comment on issues about which they feel strongly. In practice the additional comment section on a questionnaire often points to areas where patient satisfaction can be improved, sometimes very simply, and draws attention to aspects of their care which may not have been considered.

Staff should also be encouraged to anonymously complete questionnaires that relate to how they are managed, their working environment and job satisfaction so that common trends can be discussed and addressed.

Quality objectives

Continuous improvement is one of the main aspects of quality management, and one of the ways to help a clinic improve is for every area to have one or two specific goals to be achieved over the year. These quality objectives should be linked to fulfilling the promises made in the quality policy, for example, a plan to reduce multiple pregnancy rates or improve communication with referring consultants.

Quality indicators

The clinic will need to set up a system for monitoring its performance in relation to the quality of patient care. The frequency of the monitoring will depend on the area being evaluated, and the HFEA currently suggests which areas should be covered. Examples include: counselling, provision of information and consents. These areas may be assessed by a combination of auditing, staff competency checks and review of data, to ensure that standards are being maintained.

Included in the quality indicators are some critical areas that the clinic will want to monitor on a more frequent basis in order to quickly detect any downward trend, which if it continued could adversely affect the success and efficiency of the clinic. These may be referred to as Key Performance Indicators (KPIs).

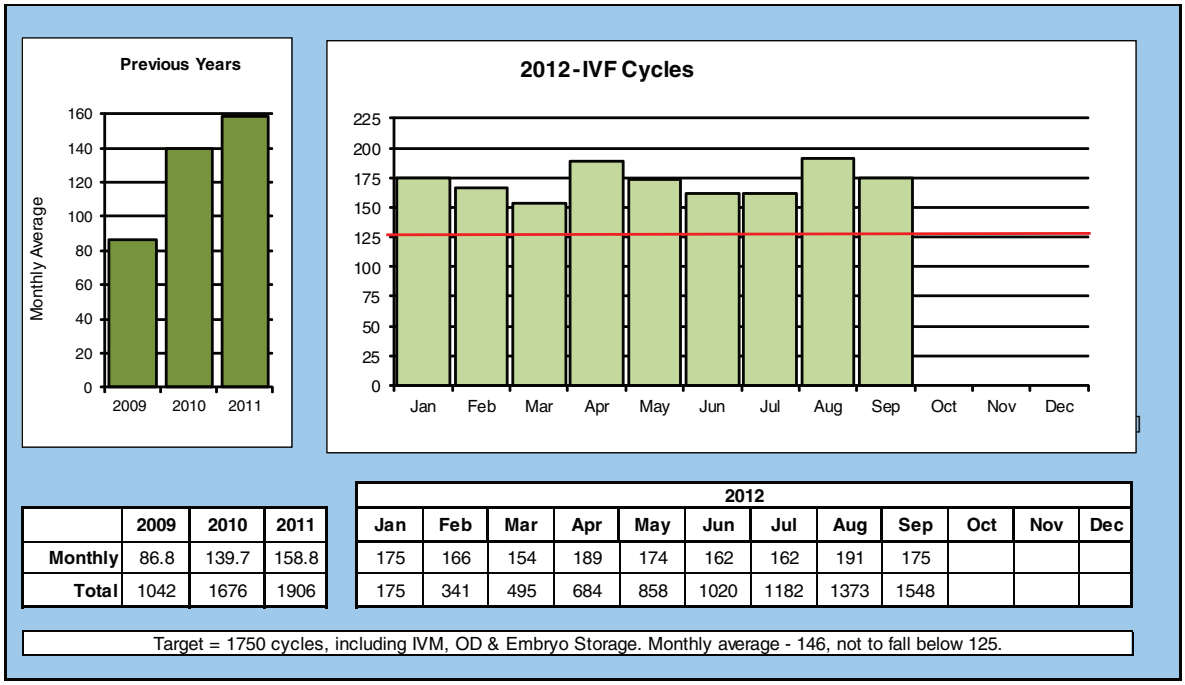
Key Performance Indicators (KPIs)

KPIs are usually monitored on a monthly basis to act as an early warning system. Minimum targets for each indicator are set and if they are not met, early investigation can be carried out and appropriate action taken. A good KPI will be measurable, current and actionable. For example, while the livebirth rate is important, by the time the results are available it is too late to be useful. Targets should be set by senior managers on an annual basis; these should be realistic, and are usually based on previous performance figures. The results are then regularly reviewed and presented to all staff, with clear procedures to follow if there are target failures. Presenting the data in graph format allows any trends to be clearly seen, as shown in Figure 21.1. The number of KPIs used will depend on the preferences of the clinic, but in most cases more than ten will become difficult to manage, reducing their usefulness.

Examples of KPIs are:

- Number of patients referred to clinic
- Number of cycles performed
- Fertilization rate
- Embryo cleavage rate
- Embryo quality
- Implantation rate
- Pregnancy rate

Using cleavage rate as an example, the target may be set at 95%. Then if the cleavage rate was only 92% it would trigger an investigation, looking at areas such as incubator usage and culture media batches. If a problem was identified it could be detected, and hopefully rectified, much sooner in this way than if only pregnancy rates were being monitored.



	2009	2010	2011
Monthly	86.8	139.7	158.8
Total	1042	1676	1906

2012												
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
175	166	154	189	174	162	162	191	175			
175	341	495	684	858	1020	1182	1373	1548			

Target = 1750 cycles, including IVM, OD & Embryo Storage. Monthly average - 146, not to fall below 125.

Figure 21.1 An example of a Key Performance Indicator chart for the number of IVF cycles performed. This would be updated on a monthly basis.

Validation of equipment and processes

All equipment that is used for critical processes must be validated to ensure that it meets all documented requirements and is functioning to the required standards. All the processes involved in the validation process of each piece must be carried out by appropriately trained personnel and every step documented. The most common items of equipment in the laboratory that will require validation are:

- Incubators
- Heated surfaces
- Safety cabinets (flow hoods)
- Heating blocks
- Refrigerators and freezers
- Controlled rate cryomachines

When equipment is being considered for purchase it is helpful to write a User Requirement Specification (URS) as part of the purchasing contract. This should specify exactly what is expected from the piece of equipment in relation to its proposed function in the laboratory, for example with an incubator this should include the normal operating temperature and the acceptable variation from this. There may also be specifications for CO_2, O_2, internal alarms and access points for external monitoring. All these points should be laid out and agreed with the supplier prior to signing any order form.

More complex equipment, such as flow hoods and incubators, will normally be installed and commissioned by the suppliers, and this should be a requirement specified in the URS. At this point any checks that are to be carried out in relation to correct installation known as the Installation Qualification (IQ) should be addressed. Examples of points covered here are:

- Is the equipment uniquely identifiable by a serial number?
- Has it been installed in the correct position and connected to the required services?
- Have commissioning documents and operating manual been supplied?

Once these points have been satisfactorily answered, the Operational Qualification (OQ) can take place to ensure that all components function correctly. The actual checks carried out will depend on the specific piece of equipment being validated. The OQ will check

all the functions of the equipment and include the simulation of faults, for example, does the alarm sound if the temperature is too high, and what is the effect of a power failure?

Many items of equipment in the laboratory will require temperature mapping to check the temperature measurement in different areas within the equipment. Different areas of an incubator or a heated surface for example, are likely to run at slightly different temperatures. Each item of equipment will have different acceptance criteria, which must also be documented. The last part of the OQ involves ensuring that there has been adequate internal staff training in the operation and maintenance of the equipment and that an internal SOP has been created describing how this is carried out. In the UK, the Association of Clinical Embryologists provides its members with templates to help with the validation process [2].

If any non-conformance is identified at any stage, this must be documented and any potential impact assessed. If the deviations are deemed to have negligible impact on the gametes or embryos then the equipment may be given interim approval until they have been resolved. Serious non-conformances that could have a critical effect on gametes or embryos will obviously result in the equipment being unusable until the problem has been rectified.

Older equipment must also be validated, although some points will be omitted, such as the installation and commissioning checks. Any equipment that has been taken out of commission for repairs must also undergo operational checks before it is re-authorized for use.

Process validation

The critical processes carried out in the clinic must also be validated to ensure that they are appropriate, effective, reproducible and safe. The SOPs must be reviewed and compared with published protocols and results to ensure that they conform to any relevant professional guidelines and regulations. Then it must be confirmed that any member of staff carrying out a specific procedure is fully trained and competent, and that any equipment used in this procedure has been reviewed and validated where appropriate. Finally, the results of each procedure are analyzed to ensure that the required standards are being consistently met. This analysis will usually be retrospective, but ongoing monitoring may be useful if a new procedure is being introduced and there is need for reassurance that it is effective.

Monitoring and measuring of equipment

All equipment in the laboratory must be independently monitored to ensure that it is functioning correctly. It is not enough to accept that external displays (e.g. for temperature) are accurate; they must be independently monitored using devices that have themselves been correctly calibrated. The acceptable variations in each parameter should be documented, as well as the required frequency of measuring and a description of how the results are to be assessed. The acceptable limits will be set depending on what is biologically optimal as well as consideration of the built-in limits of the equipment. The frequency of the testing will be determined by how critical the equipment is. In order to ensure that the external measuring devices are accurate, they must be regularly calibrated against national standards by a certified laboratory and have documentation to verify this.

For example, an incubator being used to culture embryos at 37°C should be set at that temperature and a tolerated variation may be ± 0.5°C, as the incubator is unlikely to be able to function more accurately than this. The temperature for this piece of equipment should be monitored at least daily, but many laboratories now use electronic continuous measuring devices which can take readings once every minute. The CO_2 levels in the incubator will also need to be checked using an external device at least once a week, although again this can be linked to a continuous measuring system. The output from these instruments can then be either downloaded on a daily basis or connected to a computer system for ongoing analysis. The monitoring network can also be connected to an alarm system, allowing immediate notification of any problems so that prompt action can be taken. In the UK, having critical equipment such as incubators and liquid nitrogen storage tanks containing gametes or embryos connected to an external alarm system is a requirement of the HFEA. The wireless temperature probe used for the sperm storage tank in Figure 21.2 allows it to be moved to the liquid nitrogen filling point without losing contact with the monitoring network.

The air quality of the laboratory will also require monitoring and this is particularly critical within the safety cabinets in which embryos and gametes are manipulated. Air quality monitoring in the form of microbiological contamination and airborne particulate contamination testing should be carried out on a

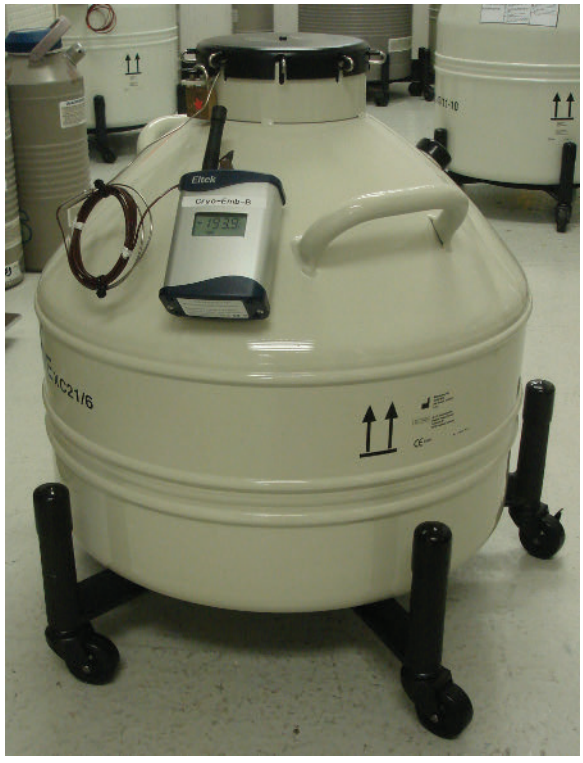


Figure 21.2 Sperm storage tank with a temperature probe which connects wirelessly to the laboratory monitoring system.

regular, usually monthly, basis. The monitoring of levels of volatile organic compounds, which can be extremely toxic to embryos, is also advisable.

Identification and traceability

Traceability of materials

In the UK, the HFEA requires that all batches of consumables that come in contact with gametes or embryos are traceable. Where possible all products used should be CE marked to demonstrate that the required European Union standards for safety, health and environmental requirements have been met. However, this does not mean that the product is necessarily suitable for embryo culture, and plasticware such as test tubes and dishes will often show variation between different manufacturers and batch lots. If possible only products specifically produced for IVF or tissue culture should be used, as these will normally have been mouse embryo tested to ensure that they do not have toxic effects.

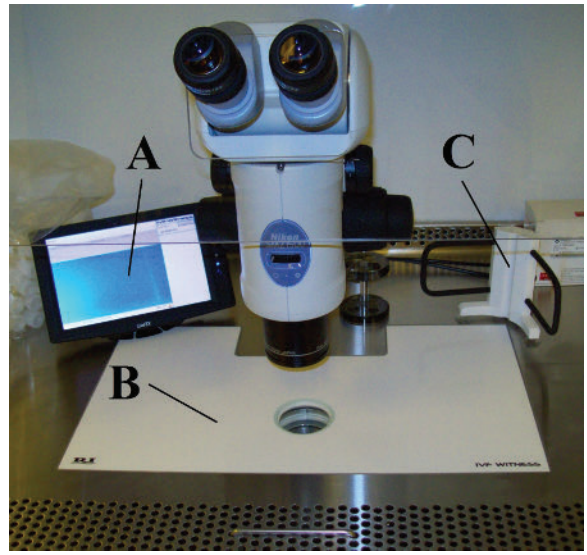


Figure 21.3 Microscope station with RFID electronic witnessing. A = Touch screen monitor for RFID system, B = Heated stage with RFID reading antenna for dishes, C = Vertical RFID reading antenna for test tubes. The image shows a system supplied by Research Instruments Ltd, Falmouth, UK.

Commercially available media is likely to have less batch variability than media made in-house, as the quality control processes are usually much more rigorous than those that could be provided by most ART laboratories. The results of the quality control processes should be made available by the manufacturer; however it is important to remember that this only covers the quality of the media when it leaves the factory. It does not take into account the conditions that it may have been exposed to in transit or storage conditions once it arrived.

Identification of gametes

It is essential that gametes and embryos are correctly identified during all laboratory processes, and this requires that robust witnessing procedures are in place. Witnessing should be contemporaneous, that is, it takes place at the time of the procedure rather than retrospectively. Witnessing may be carried out by a second member of the laboratory team; however, there is a view that this may lead to errors due to staff constantly being distracted from their own work. This has led to the introduction of electronic methods of witnessing. Electronic witnessing involves labelling the culture dishes with either barcodes or radio frequency identification (RFID) tags depending

on the system used. The barcodes or RFID signal is then read by a computer system which checks that the dishes are linked to the same patient. If this is not the case, an alarm will sound alerting staff to the error. A typical setup for a microscope station using electronic witnessing is shown in Figure 21.3.

Gametes that have been cryopreserved should be easily identifiable and at the minimum should be labelled with the patient's full name, date of freeze and a unique identifier. Documentation of the samples in storage should be properly maintained such that samples can be easily located and that those reaching legal storage limits are clearly identified.

Summary

It is important to have a regular review of the QMS, which should be done on at least an annual basis. A QMS review meeting must involve senior management and all members of the quality team. Every aspect of the QMS is discussed and any areas still needing improvement are identified. Reviewing summaries of corrective actions and audits can help to reveal any underlying trends, while discussion of changes to the volume of work or staffing levels allows their impact on all teams to be assessed and appropriate plans to be put in place. In this way the effectiveness of the QMS itself can be monitored and improved.

In summary it can be seen that implementing a QMS will help to ensure that the clinic maintains high standards and always delivers good, reliable, efficient service. In addition it provides a mechanism for continual improvement in all areas.

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Chapter 22

Regulation of assisted conception in the UK

James Lawford Davies and Alan R. Thornhill

Introduction

A comprehensive review of the regulatory framework governing assisted conception and embryo research in the UK is beyond the scope of this chapter. Instead, we provide an overview of key aspects of UK law and regulation as they relate to assisted conception, focusing on those matters of particular relevance to embryologists.

The Human Fertilisation and Embryology Authority (HFEA)

The evolution of regulation in the UK

The birth of Louise Brown in 1978 attracted extraordinary media interest and generated widespread public debate in the UK [1]. While it is possible to identify her birth as a catalyst in retrospect, legislation to regulate in vitro fertilization (IVF) and embryo research was slow to follow. IVF enjoyed relatively unfettered growth in the late 1970s and early 1980s, and ethical debate tended to be between doctors and scientists rather than parliamentarians. This began to change as political, religious and other interest groups turned their attention to embryo research, and in 1982, the Secretary of State established a 'Committee of Inquiry into Human Fertilisation and Embryology' under the Chairmanship of Dame (subsequently Baroness) Mary Warnock.

The Warnock Committee reported in June 1984 [2] and recommended that embryo research and a variety of IVF services be subject to statutory regulation and licencing, and that a new statutory regulator be set up to effect this. Although unanimous on most issues, the committee was divided over embryo research, the main objection being that it was morally wrong [3]. Notwithstanding this, the majority agreed to allow embryo research to continue, subject to strict licencing and conditions – the first elucidation of the compromise position later enshrined in law.

The Warnock Report was in fact given a frosty reception in both scientific and political circles, which prevented the government from introducing legislation at this time. There followed a series of attempts by MPs opposed to embryo research to introduce legislation prohibiting it. Most notably, Enoch Powell's 'Unborn Children (Protection) Bill' [4], which would have restricted IVF and prohibited embryo research, was passed at its first reading in the House of Commons by an overwhelming majority of 238 votes to 66. This served as a wake-up call to the research community, and the 'Progress' lobby group was set up to educate the public, MPs and Peers, gradually shifting the balance of voting in favour of research.

It was not until December 1986 that the government ventured to publish a Green Paper which essentially reiterated the recommendations of the Warnock Report, followed by a White Paper in November 1987 entitled '*Human Fertilisation and Embryology: A Framework for Legislation*' [5]. The White Paper also followed Warnock closely but it is notable that even at this late stage, the government stopped short of endorsing embryo research, instead proposing alternative clauses for Parliament to debate: one criminalizing research, the other permitting it under licence from the proposed regulatory body.

No bill followed the White Paper immediately, but the Human Fertilisation and Embryology Bill was given its first reading in the House of Lords in December 1989. After some inconclusive debates, a timely break-through in preimplantation genetic diagnosis provided a tangible example of the benefits of embryo research and helped ensure the smooth passage of the bill through

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

Parliament. The bill was passed with 303 voting in favour and just 65 against – almost the reverse of the voting on the Powell Bill just a few years earlier.

Two features of this brief history are striking. First, although the UK is now considered to be a comparatively liberal regulatory environment for IVF and embryo research, a very different model and approach were seriously contemplated as recently as 1987 which would have introduced a far more conservative, restrictive framework. Second, although today the majority of the regulatory resources are devoted to the regulation of assisted conception, the evolution of the UK framework was dominated by the controversy relating to embryo research rather than IVF.

The 1990 Act

The Human Fertilisation and Embryology Act 1990 (the 1990 Act) received Royal Assent on 1 November 1990, and the Human Fertilisation and Embryology Authority (the HFEA) opened its doors on 1 August 1991. The 1990 Act provides that the membership of the HFEA should include at least one medical practitioner and that at least half should be lay members, all serving under a lay chair and deputy chair. At the time of writing the HFEA has 12 members, only one of whom is a clinical embryologist.

The Authority members are supported by a large executive arm, and most licensed centres' day-to-day contact with the HFEA will be with the executive staff. Over time, a number of the functions of the Authority members have been delegated to the HFEA Executive, including certain licencing functions, discussed below.

The 1990 Act gives the HFEA a number of specific functions which include:

- The licencing of treatment services, the storage of gametes and embryos, and research on embryos (discussed below);
- The inspection of premises and activities carried out under a licence;
- The maintenance of a Code of Practice giving guidance about the 'proper conduct' of licensed activities (now in its 8th edition);
- The investigation of serious adverse events and reactions; and
- The maintenance of a register of information about licenced treatment services.

In addition to these, the HFEA have more general functions as follows: to keep under review information about the activities governed by the 1990 Act, to publicize the services of the HFEA licence, and to provide advice and information to licensed centres, patients and donors – much of which is achieved through the HFEA website [6].

HFEA licencing and inspection

The 1990 Act provides that certain activities must take place under a licence granted by the HFEA, including:

- The creation and use of human embryos in vitro;
- The storage of gametes and embryos;
- The storage and use of donated gametes and embryos;
- The procurement and processing of gametes and embryos; and
- The use of human embryos in research.

It is a criminal offence to perform any of these activities without a licence, punishable with a fine, imprisonment or both.

The 1990 Act enables the HFEA to grant four categories of licence:

- Licences for treatment (including the creation of embryos in vitro and 'placing' an embryo in a woman);
- Licences for non-medical fertility services (the processing and distribution of sperm for commercial purposes);
- Licences for storage (whether for embryos, gametes or both); and
- Licences for research (including both the creation of embryos in vitro for research purposes, and the storage and use of embryos for the purposes of a research project).

Whereas a research licence may be granted for a period of up to three years, the other three categories of licence may be granted for up to four years (though in practice the HFEA often grants shorter-term licences where it deems it necessary for centres to be subject to more regular scrutiny). At the time of writing, the HFEA website indicates that there are 116 treatment licences in the UK, most of which include storage, and 27 research licences.

In a move intended to expedite decision making, responsibility for determining 'routine' licencing matters has recently been delegated by the Authority to an Executive Licencing Panel consisting of three members of the HFEA executive staff. Novel, complex or controversial decisions will still be considered by a Licence Committee (or Research Licence Committee)

made up of Authority members. Another important recent change disqualifies those Authority members currently employed at licenced treatment centres from sitting on a Licence Committee when reviewing treatment licence applications from other centres owing to the clear conflict of interest this presents.

In order to obtain a new treatment or storage licence from the HFEA, or to renew an existing licence, a fee must be paid and written application made to the HFEA executive. Among other things, this application must identify the individual who will hold the position of statutory 'Person Responsible' under the licence – a role that carries with it significant duties and implications under the 1990 Act [7]. Non-compliance with the conditions of a centre's licence, or any failure by the person responsible to discharge their supervisory functions over the activities authorized by a licence may, where appropriate, result in the revocation of the licence or variation of its terms [8]. It follows that a centre's choice of candidate for the role (which must be held by a named individual) is important and should be made carefully. Prospective candidates for the role must also be approved by the HFEA and must complete an assessment.

Following receipt of a satisfactory application, the HFEA executive will arrange for an inspection of the centre's premises. A report of this inspection is then passed to the Executive Licencing Panel for consideration, whereupon there are three possible outcomes:

i. The licence is granted or renewed;
ii. It is proposed that the licence be granted or renewed subject to conditions specific to the centre (i.e. in addition to the conditions that apply to all centres); or
iii. It is proposed that the licence is refused.

Providing the centre notifies the HFEA executive within 28 days of the decision, it may make written or oral representations to the HFEA Licence Committee in relation to a proposal to refuse to grant or renew a licence, or a proposal to impose conditions upon a licence. If these representations are unsuccessful, there is a further right of appeal to the HFEA Appeal Committee, and beyond that to the High Court on a point of law.

Licences may be granted for periods of up to four years, during which time the executive conducts interim inspections and occasional unannounced inspections as it sees fit. Centres are also obliged to report adverse incidents and 'near misses' (including but not limited to harm to or loss of gametes or embryos, confidentiality breaches, mis-labelling and, more recently, severe ovarian hyperstimulation) to the HFEA within one working day, which can in turn prompt inspections during the lifetime of a centre's licence.

HFEA research regulation

Although most HFEA activity is devoted to the regulation of IVF treatment, the licencing of embryo research has long remained the most controversial aspect of its work.

The 1990 Act makes specific provision for the use of human embryos for certain research purposes conducted under a licence granted by the HFEA. Applicants for a research licence must demonstrate that their proposed research is either necessary or desirable for one or more of these purposes, and that it is necessary for them to use human embryos (as opposed to another source) for their study. The legislation originally recognized five legitimate purposes for embryo research, which primarily reflected the potential uses of embryos in research relating to human reproduction at the time. This continued without change until the advent of human embryonic stem cell research as a potential source of knowledge about human disease and disorders.

In 1999, the Chief Medical Officer's Expert Group reviewed the evidence of the potential risks and benefits of embryo research for therapeutic purposes, together with cloning technologies. Their report, known as the 'Donaldson Report', recommended that research using human embryos (whether created by IVF or cell nuclear replacement) to increase understanding about human disease and disorders and their cell-based treatments should be permitted [9]. The government accepted these recommendations and new regulations were adopted on 24 January 2001, adding three further statutory research purposes which reflected the potential of stem cell research and cell nuclear replacement [10]. These were consolidated in 2008, resulting in the following list of eight research purposes:

(a) increasing knowledge about serious disease or other serious medical conditions,
(b) developing treatments for serious disease or other serious medical conditions,
(c) increasing knowledge about the causes of any congenital disease or congenital medical condition that does not fall within paragraph (a),
(d) promoting advances in the treatment of infertility,

(e) increasing knowledge about the causes of miscarriage,
(f) developing more effective techniques of contraception,
(g) developing methods for detecting the presence of gene, chromosome or mitochondrion abnormalities in embryos before implantation, or
(h) increasing knowledge about the development of embryos.

A number of the HFEA's decisions to grant licences for embryo research have been controversial and some have been challenged through litigation, albeit unsuccessfully. Indeed, the judgments in favour of the HFEA have served to bolster their role in regulating novel areas of research such as cell nuclear replacement and – more recently – the creation of human-animal hybrid embryos for research purposes [11]. Both these techniques were the subject of litigation brought by groups fundamentally opposed to such research. They argued that embryos created through cell nuclear replacement and hybrid embryos fell outside the definition of an 'embryo' in the 1990 Act and, as such, their creation and use for research purposes could not be licenced by the HFEA. The courts have not accepted these arguments, finding instead that although neither technique was specifically addressed in the 1990 Act as originally drafted, Parliament would not have intended such research to fall outside the HFEA's remit.

The 2008 Act and the future of the HFEA

Despite the failure of legal challenges to the HFEA's authority in relation to treatment and research regulation, by 2006 the government was persuaded that a review of the legislative framework was appropriate. Following a consultation process, a White Paper was published which confirmed that the government would not revisit the model of regulation 'whereby Parliament sets the prohibitions and parameters within which a statutory authority licenses activities' [12]. These proposals in turn led to the Human Fertilisation and Embryology Act 2008 ('the 2008 Act') which served to amend and update (but not replace) the 1990 Act. The 2008 Act explicitly addresses a number of new procedures and technologies which have emerged since the 1990 Act was drafted, such as preimplantation genetics and the relatively new areas of embryo research discussed above. The 2008 Act also made a number of notable amendments to the parenthood provisions in the legislation.

The new provisions of the 2008 Act were implemented stage by stage, with the last coming into force in April 2010. No sooner had this revision of the regulatory framework been completed than a new government was elected which announced in July 2010 that it would radically reduce the number of arm's-length bodies in an effort to make savings and improve efficacy and efficiency [13]. It is suggested that the functions of the HFEA be allocated to three other bodies:

(a) The licencing and regulation of fertility clinics would be transferred to the Care Quality Commission (CQC) – an independent regulator of health and social care provided within public and private entities;
(b) The licencing and regulation of research would be transferred to a new research regulator which would be responsible for the regulation and governance of all medical research; and
(c) The data collection and retention functions would pass to the Health and Social Care Information Centre – an independent source of health and social care information.

The indicative timetable for the implementation of these proposals was that the HFEA be abolished and its functions transferred elsewhere in April 2013. However, in a recent eleventh-hour U-turn the government announced that the HFEA would not be abolished and instead would retain its functions for the foreseeable future.

This is not the first time that reform of the HFEA has been suggested: the merging of the HFEA with the Human Tissue Authority (HTA) to create a single regulator was first mooted in 2008 but scrapped after the sector expressed concern about the proposals. It therefore remains to be seen whether the renewed suggestion that HFEA works more closely or merges with the HTA will survive scrutiny and consultation. What seems certain, however, is that the regulation of assisted conception and embryo research in the UK will continue to evolve and present novel and unpredictable challenges to legislators for years to come.

Key aspects of regulation of assisted conception

Consent

In many respects, consent is the keystone of the legal framework for licensed assisted conception treatment in the UK. As a result, however, it has also been a source of disputes and litigation: a significant number of the court cases relating to assisted conception in the UK have been linked to consent in one way or another.

An early but notable illustration of the importance of consent under the 1990 Act arose in the well-known case brought by Diane Blood [14]. Mrs Blood wished to be allowed to use sperm removed from her comatose husband before he died. The couple had been trying to conceive before he fell ill, but Mr Blood had provided no written consent or authorization for the removal or use of his sperm. In light of this, the HFEA declined to allow Mrs Blood to be treated using the sperm, whether in the UK or abroad, so she commenced legal proceedings against the HFEA. In the Court of Appeal, Lord Woolf considered the structure of the 1990 Act and the operation of the consent provisions, setting out the following analysis of the law:

- No person shall store or (in the course of providing treatment services) use sperm except in pursuance of a licence;
- Compliance with Schedule 3 of the 1990 Act is a condition of every licence;
- Schedule 3, entitled 'Consents to the use of gametes and embryos', requires amongst other things that:
  - Consent must be in writing and must not have been withdrawn;
  - Consent to storage must specify the maximum period of storage and state what is to be done with the gametes and embryos if the person dies or loses capacity; and
  - Before a person gives consent they must be offered counselling and be given such relevant information as is proper.
- Schedule 3 also provides that gametes must not be used or stored unless there is an effective consent to them being used and stored, and they are used and stored in accordance with the terms of that consent.

With this in mind, Lord Woolf concluded that '[t]he absence of the necessary consent means that both the treatment of Mrs Blood and the storage of Mr Blood's sperm would be prohibited by the Act of 1990'. Furthermore, Lord Woolf found that a technical criminal offence was committed as a result of the storage of the sperm, though he emphasized that there was no question of prosecution since the clinic had acted in close consultation with the HFEA throughout. In this respect, however, the judgment does serve to highlight the potential significance of missing, ineffective or incorrect consent forms.

The Court of Appeal was more supportive of Mrs Blood's arguments relating to the proposed export of the sperm and, following reconsideration of her application by the HFEA, she was permitted to export the samples to Belgium where she was successfully treated. Other litigants have been less successful in challenging the rigidity of the 1990 Act's consent provisions. In 2002, the Court of Appeal came to consider the request of Mrs U who wished to be allowed to use the sperm of her late husband notwithstanding that he had amended his consent form so as to withdraw his consent to posthumous use [15]. Mrs U contended that her husband had been subject to undue influence to amend his consent form by their treating clinic, which had a policy of not providing posthumous treatment due to ethical concerns. The Court did not accept her argument and again reiterated the emphasis on the importance of consent in the scheme of the 1990 Act. As Lady Justice Hale put it:

> *There is a natural human temptation to try to bend the law so as to give [Mrs U] what she wants and what she truly believes her husband would have wanted. But we have to resist it.*

The cases of both Mrs U and Mrs Blood came to be considered and applied in what is perhaps the most well known of this line of cases relating to consent – that of this line of cases relating to Natallie Evans [16]. Prior to having her ovaries removed, Ms Evans had six embryos created and stored using her eggs and the sperm of her partner, Howard Johnston. The couple subsequently split and Mr Johnston withdrew his consent to the continued storage and use of the embryos. The 1990 Act permits the withdrawal of consent by either partner at any point up until embryos are used, and this was printed at the top of the consent forms the couple had signed. Following Mr Johnston's provision of notice of his withdrawal, the clinic was therefore required as a matter of law to remove the embryos from storage. Ms Evans sought to challenge this, arguing (among other things) that the operation of the 1990 Act in this manner contravened her human rights, in particular her right to respect for her private life.

Ms Evans was unsuccessful, both in the UK courts and in the European Court of Human Rights (ECtHR). In the UK Court of Appeal, their Lordships maintained the rigorous stance seen in the earlier cases described above in their finding that:

> *the clear policy of the Act is to ensure continuing consent from the commencement of treatment to the point of implant. Consent may be given subject to conditions. Consent may be varied. Consent may be withdrawn. Against that background the court should be extremely*

> *slow to recognise or to create a principle of waiver that would conflict with the parliamentary scheme.*

The court found that Mr Johnston was entitled to withdraw his consent and further, that by permitting him to do so (thereby rendering Ms Evans unable to conceive her own genetic child), the 1990 Act did not contravene her right to respect for her private life. The reasoning behind this decision was perhaps most succinctly summed up by the Grand Chamber of the ECtHR which stated as follows:

> *As regards the balance struck between the conflicting Article 8 rights of the parties to the IVF treatment, the Grand Chamber, in common with every other court which has examined this case, has great sympathy for the applicant, who clearly desires a genetically related child above all else. However . . . it does not consider that the applicant's right to respect for the decision to become a parent in the genetic sense should be accorded greater weight than J's right to respect for his decision not to have a genetically-related child with her.*

Such rights-based reasoning may seem far removed from the routine operation of an IVF clinic, but each of the cases described above arose out of relatively familiar circumstances. They serve to highlight to clinics the importance of ensuring that patients understand the forms and information they are given, but also contemplate the implications of their decisions and future changes to their circumstances.

Status and ownership

There have been a number of recent cases in which patients have sought to claim property rights in their reproductive material. Although property rights have been asserted in relation to other tissues and cells, assisted conception in the UK has been largely free of such claims. This changed in 2009 with the case of *Yearworth and Others v North Bristol NHS Trust* [17]. The claimants were cancer patients at a hospital run by the Trust and each had sperm samples frozen and stored in the hospital's IVF unit prior to chemotherapy. As a result of an equipment failure, the samples thawed and were damaged. The men claimed that they suffered mental distress and/or psychiatric injury on learning of the loss of their sperm samples. The case went to the Court of Appeal where their Lordships agreed with the Trust's submissions that the damage to and consequential loss of the sperm samples did not constitute personal injury. However, the court went on to re-examine whether there might be a claim arising out of the fact that the samples were property owned by the men.

The court reviewed historic case law, noting that '[t]he law . . . has remained noticeably silent about parts or products of a living human body, probably because, until recently, medical science did not endow them with any value or other significance'. The court recognized that the tight regulation of clinics and the samples they store by the HFEA did serve to erode and limit the rights and control exercised by the claimants, but their Lordships held that this did not amount to an elimination of their rights. Indeed, the consent requirements of the 1990 Act provided the men with a negative control over the samples which remained absolute. For these and other reasons, the court held that the men did indeed have ownership of the sperm samples for the purpose of their claims, and further, that there was a bailment of the sperm by the men (the bailees) to the Trust (the bailor) which meant that the Trust could be liable to them in bailment.

Yearworth is undoubtedly noteworthy, not least because it has spawned a number of similar claims against IVF clinics in connection with both damaged gametes and embryos. Its implications beyond the particular circumstances of this case, however, are likely to be limited. The Court of Appeal was concerned with the status of gametes from a living body, stored under licence and intended for use by the men whose bodies had produced them. The proprietary status of reproductive tissue and cells in different circumstances (and indeed of non-reproductive tissue) will need to be considered on a case-by-case basis.

The Court of Appeal's decision in *Yearworth* does, however, create a number of legal dichotomies and dilemmas which warrant attention. First, the judgment indicates that the *purpose* for which gametes are stored will affect its proprietary status: it follows that sperm stored for treatment may be capable of ownership, while sperm stored for the purposes of a research project may not.

Second, the case only concerned gametes and the courts have previously rejected any suggestion that there could be property in an embryo. In *Evans*, above, the Court of Appeal recognized that since a fetus prior to the moment of birth does not have independent rights or interests, '[t]hus even more clearly can there be no independent rights or interests in stored embryos'. Given the numerous ethical, legal and biological distinctions to be drawn between gametes and embryos, it seems unlikely that the extant cases seeking

to assert property rights in embryos will be successful, but this is a dynamic area of law.

Third, *Yearworth* permits compensation for the owners of damaged gametes, while the same donors would be prohibited from selling their sperm samples for profit under UK law. Although the payment of gamete donors has long been a feature of US assisted conception, it has to date been frowned upon in the UK on policy grounds, echoing the approach of European law [18]. The HFEA recently reviewed its policy in this regard [19] and *Yearworth* and its sister cases may encourage a shift towards a position that is more consistent with the Court of Appeal's approach.

Finally, the Court of Appeal in *Yearworth* was not required to address the impact of their judgment on the contractual arrangements between clinics and patients. Commonly, however, patients who arrange for gametes or embryos to be stored at a clinic will enter into a

Table 22.1 Examples of specific areas of assisted conception which are covered by other regulations or under professional guidelines

Area	Professional/Regulatory body	Purpose	Scope
Clinical care	Care Quality Commission http://www.cqc.org.uk	Regulates and licences health and social care services in England. Aim to ensure standards of quality and safety maintained	In 2009, responsible for regulating 392 NHS trusts and >3000 independent (private and voluntary) providers of healthcare in England
Clinical care	General Medical Council http://www.gmc-uk.org	Registers doctors to practise medicine in the UK. Aims to protect, promote and maintain the health and safety of the public by ensuring proper standards in the practice of medicine.	Over 226 000 GMC registered doctors with a licence in UK
Embryology/ Andrology	Health Professions Council http://www.hpc-uk.org	Regulator set up to protect the public. Maintains a register of health professionals who meet standards for training, professional skills, behaviour and health. State registration	205, 000 registrants from 15 professions (from Art Therapists to Clinical scientists)
Embryology	Association of Clinical Embryologists https://www.embryologists.org.uk	Professional body of and for embryologists in the UK and overseas. Aims to promote high standards of practice in clinical embryology.	More than 750 members
Gamete donor screening	Association of Biomedical Andrologists http://www.aba.uk.net; Association of Clinical Embryologists; British Andrology Society http://www.britishandrology.org.uk; British Fertility Society http://www.britishfertilitysociety.org.uk; Royal College of Obstetricians and Gynaecologists http://www.rcog.org.uk.	Professional guidelines (published document used to inform professional members as well as HFEA Code of Practice and guidance.	UK guidelines for the medical and laboratory screening of sperm, egg and embryo donors [20]
Diagnostic testing (pathology services)	Clinical Pathology Accreditation (UK) Ltd – CPA (UK) http://www.cpa-uk.co.uk	Accreditation of Clinical Pathology Services and External Quality Assessment Schemes (EQA). Provides external audit of a laboratory's ability to provide a service of high quality by declaring a defined standard of practice, which is confirmed by peer review	More than 1000 CPA (UK) accredited medical laboratories in UK and overseas (as of 2/2011)
Counselling	British Infertility Counselling Association http://www.bica.net	Accredits infertility counsellors	158 members with 72 accredited or in assessment (as of 2/2011)
Quality Management	International Standards Organization (ISO 9001;2008) http://www.iso.org	Provide standardized framework for quality management systems designed to help organizations ensure they meet the needs of customers and other stakeholders	Global organization issuing 1064785 ISO 9001;2000/2008 certificates in 2009

contract whereby they agree to pay a fee for ongoing storage. Such contracts routinely include a clause which explains that the material will be removed from storage if the fee is not paid. While any property interests attaching to the material should not affect this agreement, it is likely that they will be relied upon in the event of any dispute. Clinics should therefore ensure that their contracts are robust.

Accreditation and other professional oversight

As the HFEA moves towards its stated goal of 'lighter touch' regulation, a greater reliance on licencing, regulation and professional guidance from other agencies and professional bodies intersecting with assisted conception is predicted. Independent of HFEA regulation, most of the routine practice of assisted conception is currently covered by other professional standards and agencies including clinical aspects of care, diagnostic testing, embryology, donor screening, quality management and counselling (see Table 22.1).

Concluding remarks

The regulation of assisted conception – a relatively young and dynamic branch of medicine – is generally regarded within the field as a positive factor in that it

Table 22.2 Examples of current specific areas of HFEA regulation with unintended detrimental consequences for patients

Area of practice	HFEA reference (Code of Practice 8th Edition) [21]	Description	Rationale	Consequences (short term)	Consequences (long term)
Embryo transfer	Guidance note 21.4 (a)	Transfer of IVF and ICSI embryos together is an exception and limited to 2% all transfers annually	Monitor outcomes from IVF and ICSI babies separately	Providers may avoid transferring best quality embryos together in a fresh cycle	Centres may prematurely opt for ICSI before considering IVF or IVF/ICSI
Embryo transfer	Licence condition T88	Transfer of biopsied and non-biopsied embryos together is prohibited	Monitor outcomes separately	As above	
Embryo transfer	Guidance note 7.3(b)	Transfer of >2 embryos in women < 40 years (regardless of fresh or frozen cycle)	Avoid high multiple pregnancy rates	Does not account for (i) biological variance among women < 40 yrs and may reduce pregnancy rates (ii) age of woman at the time of freeze but only at the time of transfer (illogical)	May interfere with patient autonomy and clinician-patient relationship. May encourage reproductive tourism as patients seek less restrictive treatment overseas.
Gamete donation	Licence condition T55	Sperm or egg donors who are carriers of a serious genetic condition should not be used	Reduce risk of transmitting inherited disorder from gamete donor	Further reduction in pool of eligible gamete donors; anxiety and disappointment in an otherwise healthy donor	Possible disincentive to thoroughly screen potential donors in future (as increased genome-wide testing becomes affordable)
Gamete donation	Guidance notes 12.4; HFE Act 1990 (as amended) [22] General conditions 12 (1) e	Egg-sharing permitted but payment of egg donors prohibited by law	Benefits in kind and donation from a fully informed IVF patient more 'acceptable' than payment to a donor	Egg sharing more frequent than egg donation. Eggs from infertile patients with potentially worse outcomes for both egg sharer and recipient. Potential disappointment for unsuccessful sharers with successful recipients	Increase in reproductive tourism as recipients seek treatment overseas. Potential increase in multiple births.

can serve to reassure the public in what are often controversial and ethically sensitive areas. The regulatory framework also provides a degree of clarity for clinical practice and research alike, serving to protect those involved to a certain extent, and encouraging good clinical and scientific practice. Recent changes in the law also mean that the UK is able to offer a regulatory environment that is relatively supportive of scientific innovation and progress. In order to operate effectively, however, UK regulation needs to continue to be adaptable and accommodate the inevitable further developments in the field. The implementation of regulation should be proportionate, particularly in light of the fact that assisted conception has now resulted in the birth of millions of healthy children and is comparatively low risk. Wherever possible, the regulator should be impartial, objective, expert and efficient in its approach to licencing and inspection. These qualities have not been consistently exhibited in the UK, and it remains to be seen whether the upcoming changes to the regulatory landscape will have a positive impact in this regard.

Lastly, it is important for regulation and policy to strike a balance between cost-effective regulation on the one hand, and interference with commercial interests, patient-clinician relationships and the daily operations of a centre on the other. For example, a number of areas of assisted conception are now subject to proscriptive and precise 'rules' enshrined as guidance or licence conditions by the regulator. While introduced with good intentions, these 'rules' can have unintended short- and long-term consequences which may be detrimental to optimal patient care and the delivery of high-quality, cost-effective services (see Table 22.2). It is for the UK and other countries to attempt to get the regulatory balance right by keeping the needs of the patient at the forefront of decision making.

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Chapter 23

Fundamental laboratory skills for clinical embryologists

Celine Jones, Junaid Kashir, Bianka Seres, Jane Chan, Kornelia Ewald and Kevin Coward

Introduction

Laboratories are utilized in nearly all fields of science and follow strict rules of conduct. In each laboratory, a specific hierarchy is in place such that personnel in the laboratory can work safely and efficiently.

Familiarizing yourself with personnel, laboratory rules and setting is essential to ensure a safe and productive working environment. Accidents can be prevented before they occur by adopting the correct attitude in the workplace and by following strict rules of conduct. These rules should be presented to any new members of staff and visitors. They explain safety procedures, laboratory hierarchy and policies for complaints and emergencies. In this chapter, we will describe some essential rules of conduct needed for safe and efficient work in a laboratory environment, highlighting what we believe are the most fundamental laboratory skills, including solution chemistry, commonly used equipment, liquid handling and the application of microscopy. Our intention is to indentify the basic skill base required to work in a clinical embryology laboratory, and to provide a solid foundation for future development.

Organization of an embryology unit

An embryology unit is headed by a director who is responsible to a variety of regulatory and ethical bodies, and leads a team of specialist managers who direct finance, administration, quality management, medical and laboratory personnel and nursing teams. These positions are, in turn, responsible for all staff within the unit. Embryology units are sometimes linked with university departments, with senior unit staff holding dual affiliations with the two institutions. This permits extensive collaboration between clinical and academic staff on such matters as clinical diagnostics, graduate and postgraduate education and scientific research. Collectively, these arrangements serve to ensure that the clinical team is constantly informed of relevant scientific developments from the research sector. A typical example is that of the Institute of Reproductive Sciences (IRS) in Oxford, a building occupied by the Oxford Fertility Unit, along with representatives from the University of Oxford, and a private company, Reprogenetics UK. Collectively, the IRS provides clinical diagnostics and treatment, genetic diagnostic technology, education and research all under one roof. This ensures that clinical and scientific staff members liaise effectively, and provides a highly motivating and inspiring learning environment for graduate and postgraduate students, such as those registered for the Oxford MSc in Clinical Embryology, which operates from dedicated laboratory and teaching facilities within the IRS. Interaction within the different operating units of the IRS is complex and operates at many different levels. Consequently, in order to maximize efficiency it is important for all staff to develop a deep understanding of how the network operates. For a schematic summarizing these networks, please refer to Figure 23.1.

Communication among workers

It is essential to have a system in place to permit efficient and timely communication to all laboratory staff and students. Electronic mail is a good way of disseminating important information to everyone, for example, the notification of obligatory meetings and routine service updates. However, regular face-to-face meetings should be compulsory for all managerial, research and technical staff to allow discussion of regulatory changes, ethical requirements, critical laboratory issues such as laboratory cleanliness and safety,

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

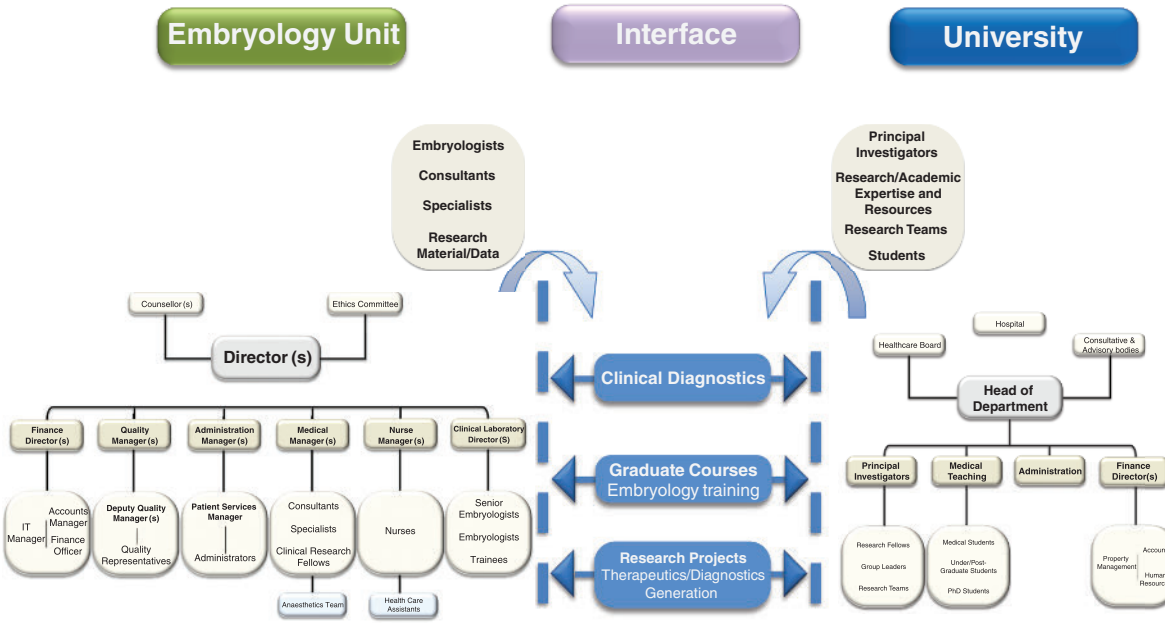

Figure 23.1 Organogram of the typical interactions between an embryology unit and an academic university environment using the relationship between Oxford Fertility Unit and the University of Oxford as an example.

the state of equipment, research progress and technique development.

A clinical embryologist should possess excellent communications skills. All embryologists must possess good verbal communication skills to be able to discuss clinical matters with patients and to present results to colleagues. An understanding of administrative procedures is also essential as standardized and highly detailed forms need to be completed by embryologists in the course of their work. White boards in each section of a laboratory are a good way of communicating local information, for example, items that need to be ordered, holiday planning and notification of damaged equipment. It may be more efficient to display information referring to apparatus on the machine itself if there is more than one piece of identical equipment in the laboratory. If unique machines are non-operational, then an email to all personnel may be better.

Safety procedures

Consideration of safety, for oneself and other laboratory staff, is essential. Many factors have to be taken into account, as an individual can easily put herself and/or others at risk if safety protocols are not followed. Both long- and short-term work must be considered when creating safety protocols, including the expansion of laboratory work and research teams. Safety policies should be given to any new employee or student before they commence laboratory work and should include instructions on how to proceed in case of fire, where to obtain first aid, waste disposal protocols, health check requirements and how to report accidents. Each individual should be aware of all laboratory policies, their own safety responsibilities, and should understand how specific policies work within the laboratory. Failing to follow the policies of the workplace could be hazardous but could also drastically reduce the productivity of the laboratory.

Each procedure in a workplace must be risk assessed, which means that all potential hazards have been assessed and a written record (Risk Assessment Form) created that identifies each risk and the controls necessary to mitigate these risks. The health and safety executive provides guidance of how to assess risks in a workplace [1]. Control of substance hazardous to health (COSHH) describes hazards, control of exposure, emergency procedures and the disposal of substances. Manufacturers are required to provide information about the risks involved in the use of their equipment (Equipment RA) or chemicals (Chemical RA *together with* COSHH analysis). For example, chemical suppliers provide a material safety

data sheet (MSDS) to describe the physical data, health hazards and disposal procedures for each individual chemical they sell.

Protective clothing

It is important to follow laboratory dress code. Colour-coded outfits are sometimes used to distinguish staff, for example to identify nurses from embryologists and technicians. For certain duties, disposable laboratory garments may be available, such as when handling blood. In these cases a plastic apron should be worn on top of the laboratory dress code which will prevent spoilage in the case of spillage. Adopting the dress code of a laboratory will not only prevent possible damage to the laboratory workers' own clothes but will also avoid cross-contamination caused by taking spoiled clothes back to private homes. Laboratory garments are usually laundered by specialist external companies hired by the unit, and replaced when worn out or damaged. Sensible footwear must be worn at all times and embryologists have specific laboratory shoes or overshoes to wear whenever in regulated clinical areas.

Eye injuries can occur in laboratories and it is often compulsory to wear safety glasses when the procedures being undertaken involve splashing. Face visors are also used when necessary, for example, when dispensing liquid nitrogen (N_2).

Various types of laboratory gloves must be used depending on the task at hand, for example heat-resistant gloves, ultra-cold gloves and latex gloves for everyday procedures. It is becoming increasingly common for laboratories to use specialized hypoallergenic gloves, to prevent allergic reactions to latex gloves. Consequently, most laboratories now use latex-free gloves made from nitrile products.

Signage/labelling in a laboratory

Signage is a very important part of laboratory safety as it alerts laboratory users and visitors of specific hazards in various locations within a building. However, it should be noted that signage ***does not*** replace basic laboratory training. As well as being useful for hazard warnings, signs can also be an omnipresent reminder for permanent staff (e.g. to prevent drinking and eating in controlled areas). Signs are an extremely useful part of everyday life, although a laboratory should not become overcrowded as may result in confusion.

Labelling bottles, boxes and cupboards is a very good way of preventing inadvertent loss and experimental error. Appropriate signage also promotes efficient economics, since it prevents the overstocking of common laboratory items. Efficient organization of the workplace is essential. For example, unused or infrequently used items may be stored on top shelves, while everyday equipment and goods should be within easy reach of personnel. An organized laboratory will save time for employees and students and will also save money.

Dating chemicals and solutions is also essential, as these may eventually lose potency (for example if they are bio- or photo-degradable). If the chemical is a laboratory-prepared solution or buffer, adding the researcher's name and the exact content of the bottle will prevent potential harm to personnel or mistakes in laboratory procedures. Finally, a cluttered lab is potentially dangerous and can be stressful for staff members. For example, boxes and unfiled paper can create trip and fall hazards when stored on the floor; these should be stored on an appropriate shelf, or within an appropriate cupboard or cabinet.

Handling laboratory equipment

Before workers commence using apparatus in a laboratory, appropriate training must be received and fully understood. Competency testing may be deployed to ensure compliance. Equipment may be delicate, susceptible to damage if improperly used and expensive to replace.

Centrifuge techniques

Centrifuge techniques are used routinely in embryology labs and permit the separation of particles of different mass and size, for example the separation of dead and motile sperm during gradient sperm preparation.

Many types of centrifuge are available on the commercial market, incorporating various sizes and speeds. The most commonly used centrifuges adopt bench-top, micro-, high-speed and ultra-platforms. Different centrifuge systems exert differential *g*-forces and may possess different types of rotor to accommodate differing tubes and volume capacities, or may be able to operate over a range of temperatures (e.g. cryogenic centrifuge). The type of rotor used is very important and depends upon the specific nature of the laboratory procedure being undertaken. Fixed angle rotors

are the fastest at precipitating particles, have the fewest moving parts and are therefore less prone to mechanical failure. However, these rotors may cause damage to separated particles as they are forced down the side of the tube. Swinging bucket rotors are very versatile since they can accommodate tubes of many different shapes and have a much gentler effect upon the separated particles. However, these rotors have many more moving parts and are generally slower than fixed angle centrifuges. Before a centrifuge can be operated, an appropriate counterweight must be used to balance the mass of the sample and sample tube, and the rotor cover should be closed. Centrifugal speed is given in gravitation force (*g*), or *Relative Centrifugal Force* (RCF), the latter being synonymous with revolutions per minute (rpm). For individual protocols, the *g*-force unit is generally used and may be calculated from rpm as follows: $g = 1.12 \times 10^{-6} \times$ rotor radius (mm) $\times$ rpm^2. It is important to note that RPM varies between centrifuges, and is dependent upon rotor size, rotor type and centrifuge model.

Decontamination and disinfection in the laboratory

Sterilization is used to destroy microbial life. This is best achieved by physical methods like steam (autoclave), dry heat (baking oven), ultra-violet (UV) light or by membrane filtration. Disinfection, however, is used to destroy pathogenic organisms. Chemicals such as ethanol are often employed for this purpose. Autoclaves kill bacteria, viruses, fungi, spores and work by applying steam under pressure. This results in irreversible coagulation of microbial proteins. Not everything may be sterilized in an autoclave and care must be taken to ensure that materials destined for autoclave sterilization can withstand the extreme temperature and steam. Errors can result in melting (plastics) and decomposition (for materials with low decomposition temperatures), hydrolyzation and oxidation (e.g. steel, iron). Baking ovens are also used for sterilization. These are often used to sterilize metallic instruments as the baking process is less corrosive to metals. UV lights are sometimes used in cabinets and certain other equipment. UV lights are often fixed within cabinets such that they are not easily accessible, and the cabinet doors often feature a security mechanism that switches off the UV light if the door is accidentally opened during a UV sterilization cycle. UV light causes thymine molecules to dimerize and can therefore cause extensive damage to DNA. Caution is also needed when using UV lights to avoid burn injury. Small volumes of liquid can be sterilized by passing through micro-filters attached to a syringe. These are very convenient and available in sterile, single-wrapped units.

Blood-borne viruses (BBV)

BBVs are viruses found within body fluids like semen, blood and vaginal secretions [1]. Clinical embryologists may, therefore, be exposed to fluids that could be infected. Direct exposure often occurs as a result of accidents involving sharp instruments, via open wounds and splashes to the face. Therefore, it is important to apply strict hygiene practice in the workplace and to cover any existing wounds by application of first aid before working with specimens. Immunization will be available via the workplace against certain BBV (for example, Hepatitis B). Eating and drinking are prohibited in the laboratory, and the potential risk for contamination is reduced by having a vigorous decontamination policy in place. Aseptic techniques are used to maintain the absence of pathogens in the clinical setting, thereby protecting both patients and workers.

Liquid handling techniques

One of the most fundamental skills in any laboratory is the ability to handle very small volumes of liquid in an accurate and reproducible manner. Anyone who has worked in a laboratory will be aware of the pipette, a small hand-held device purpose-built for handling liquids. However, using the humble laboratory pipette must not be taken lightly. It is vital that workers understand how these devices work, how to maintain accuracy and consistency, and critically, how to ensure calibration at all times. Errors in manipulating volumes can be extremely detrimental in the research laboratory, but are particularly dangerous in the clinical IVF laboratory, where maintaining a strictly regulated micro-climate for gametes and embryos is critical. To maintain consistency in terms of precision and accuracy, a high-quality laboratory pipette must be used with high-quality tips. However, these factors are rendered useless unless the laboratory worker using such devices has sufficient practical experience, is able to handle the device appropriately and understands how to maintain cleanliness. It is our intention here to introduce the basic laboratory pipette, and to provide guidance on its correct operation.

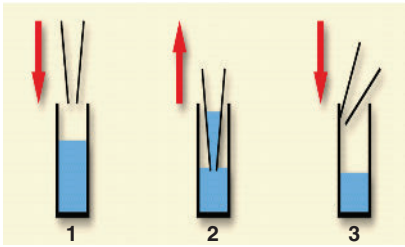

Figure 23. 2 Forward pipetting. **1**. Press down to first stop. Immerse tip a few millimetres into liquid. **2**. Release key slowly. Tip will fill up. **3**. Dispense liquid by pressing key down to first stop. Then blow out remaining liquid by pressing key down to second stop. This application is recommended for standard solutions, such as water buffers, diluted saline solutions and diluted acids/alkalis. Figure reproduced with permission from Eppendorf UK.

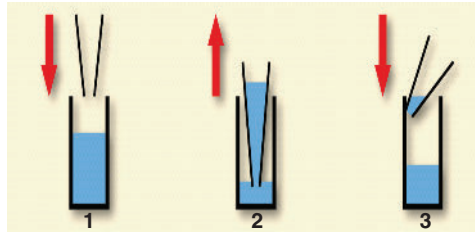

Figure 23.3 Reverse pipetting. Press down to second stop. Immerse tip a few millimetres into liquid. **2**. Release key slowly. Tip will fill up. **3**. Dispense liquid by pressing key down to first stop. Some liquid will remain in the tip. This application is recommended for viscous solutions, solutions with a high vapour pressure and wetting agents. Figure reproduced with permission from Eppendorf UK.

Dispensing techniques

The technique of forward pipetting (Fig. 23.2) is suitable for aqueous solutions which may contain low concentrations of protein or detergents. This technique involves pressing the push-button of the pipette while in the liquid and releasing it slowly by pressing the key down to the first stop. Pre-wetting of the tip improves the analysis result. When using viscous or foaming liquids, or when dispensing very small sample volumes, results may be greatly improved by a technique known as *reverse pipetting*. Here, the liquid is aspirated with blowout and dispensed without blowout. This time, the push-button is pressed to the second stop in the first instance and the key is release slowly to fill up the tip. After dispensing the liquid by pushing on the first stop, a residue of liquid remains in the plastic tip, and it is subsequently discarded or returned to the aspirating vessel (Fig. 23.3).

Optimum handling of manual pipettes

Irrespective of the dispensing technique used, the following items should be taken into consideration during pipetting [2]. In the case of air-cushion pipettes, the pipette tip should be selected so that the air cushion between the pipette piston and the surface of the liquid is as small as possible. The smaller the tip, the lower the air volume and the greater the accuracy of the results will be. When aspirating liquid, the tip should only be immersed a few millimetres into the medium. The filled tip should be moved up against the wall of the vessel to avoid residue of liquid on the outside of the tip. Pre-wetting the tip two or three times will improve the accuracy and precision of the results. Liquid should be aspirated slowly and evenly. A waiting period of 1 to 3 seconds should be allowed for the liquid to rise in the tip.

Dispensing systems function according to two different physical principles: dispensing of liquid either takes place via an air cushion or by positive displacement. These two different dispensing principles are presented below, taking piston stroke pipettes as an example. Important aspects of ergonomics are also discussed.

Air-cushion principle (air displacement)

Air-cushion pipettes consist of a piston-cylinder system which performs the actual measurement (Fig. 23.4). An air cushion separates the sample aspirated into a plastic tip from the piston inside the pipette. Upward movement of the piston produces a partial vacuum in the tip, causing the liquid to be drawn into the tip. The air cushion moved by the piston acts like an elastic spring from which the volume of liquid in the tip is suspended. Due to expansion of this air volume, the volume moved by the piston is approximately greater than the aspirated volume of liquid required. Such expansion is compensated for by a factor that takes into account the dead volume and the lift height in the tip of the pipette. The influences of temperature, air pressure and humidity must be minimized with an air-cushion pipette through design measures so that the dispensing accuracy is not impaired.

Figure 23.5 shows the design principle of the air-cushion pipette based on the rest position (1). To prepare for aspiration of the liquid (2), the push-button is pressed to the first stop (measuring stroke). The piston moves down, displacing a volume of air that corresponds to the selected aspirating volume of the liquid. To aspirate liquid (3) the pipette tip is

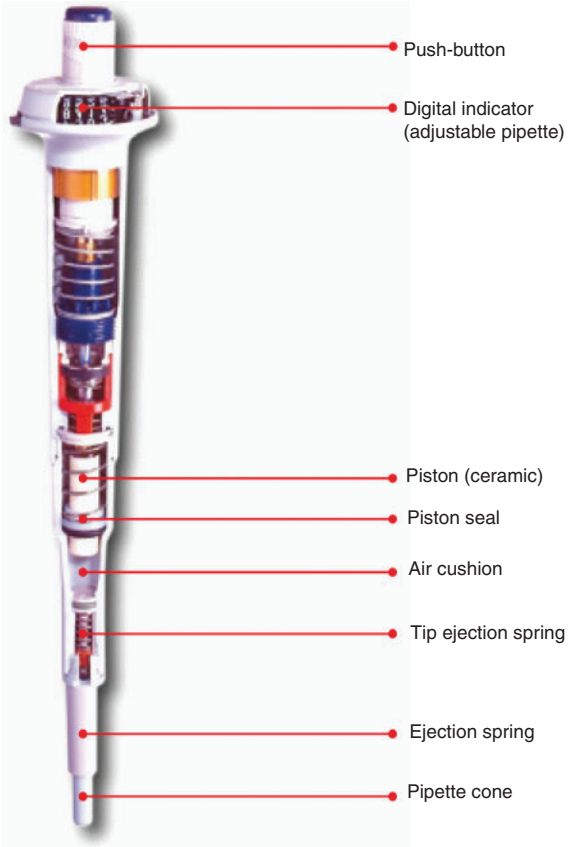

Figure 23.4 Function of an air-cushion pipetting. Figure reproduced with permission from Eppendorf UK.

immersed into the liquid vertically. As the push-button slowly moves back, a partial vacuum is created in the pipette tip, aspirating the required volume through the tip opening. To dispense liquid (4), the push-button is slowly pressed to the first stop (measuring stroke).

Principle of positive displacement

Dispensing systems operating according to the principle of positive displacement are subject to physical influences other than those occurring with the air-cushion systems described above. The effects of an air-cushion are not applicable here so that these devices are also suitable for liquids and applications that can be seen as critical in conjunction with air-cushion systems. Such applications include: liquids with high vapour pressure, high viscosity or high density and applications in molecular biology such as the polymerase chain reaction, which calls for an absence of aerosols to prevent cross-contamination. The dispensing accuracy of positive-displacement dispensing systems depends on the disposable plastic tip to an even greater extent than with air-cushion systems. Unlike the plastic tips of the air-cushion systems, the tips of the positive-displacement systems have an integrated piston, which is coupled to the piston rod of the dispensing device during the pipetting (Fig. 23.6) and the actual dispensing process.

Tips are specially designed for the use of positive-displacement systems and cannot be replaced by tips foreign to the system. Figure 23.7 shows the functioning of positive displacement. To prepare for aspiration of the liquid (1), the push-button is pressed to the first stop and the piston moves down to the corresponding position. For aspiration of the liquid (2), the pipette tip is immersed a few millimetres into the liquid vertically, the push-button is then allowed to slide back slowly, the piston moves up and the required volume of liquid is aspirated into the tip by the partial vacuum that is produced. To dispense the liquid into a vessel (3), the push-button is slowly pressed to the first stop (measuring stroke). The piston in the tip is moved down by the piston rod of the pipette, thus displacing the liquid from the tip. The push-button is held down, and the tip drawn up against the wall of the vessel. To eject the tip (4), the push-button is pressed all the way down.

Pipette tips

The pipette tip is a key component of the 'pipette' dispensing system and should never be neglected. Its shape, material properties and fit have a major impact on the accuracy of the dispensing process. It is only possible to achieve maximum precision and reliability offered by modern pipettes with perfectly manufactured pipette tips and optimum coordination between the pipette and tip. Pipette tips must be precisely shaped to ensure pipetting accuracy in the microlitre range. They must be designed so that even the tiniest drops can be dispensed accurately on the surfaces of micro test tubes.

Cleaning and inspection of pipettes

Modern quality management in the laboratory calls for the regular cleaning and inspection of dispensing systems. How often a pipette needs to be cleaned and inspected depends on actual practice, for example, the frequency of usage, number of users of the device, aggressiveness of liquids to be dispensed and, also, the acceptable error limits that have been defined by the user. The pipette should be held *vertically* when

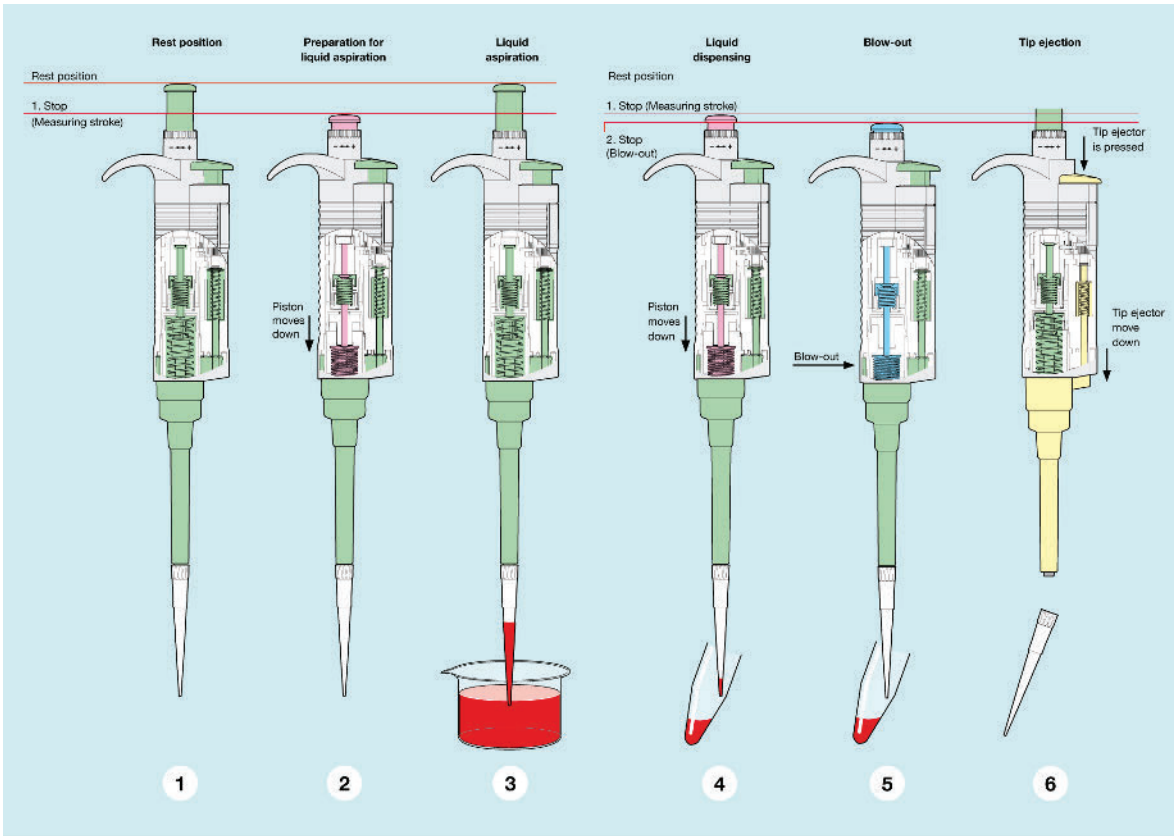

Figure 23.5 Design principle of air-cushion pipetting. The air-cushion pipette is in the rest position in (1). To prepare for aspiration of the liquid (2) the push-button is pressed to the first stop (measuring stroke). The piston moves down, displacing a volume of air that corresponds to the selected aspirating volume of the liquid. To aspirate liquid (3) the pipette tip is immersed into the liquid vertically. As the pushbutton slowly moves back, a partial vacuum is created in the pipette tip, aspirating the required volume through the tip opening. To dispense liquid (4–5), the push-button is slowly pressed to the first stop (measuring stroke). To eject the tip (6), the push-button is pressed all the way down. Figure reproduced with permission from Eppendorf UK.

aspirating the liquid. It is also recommended to immediately eject the pipette tip after usage to prevent vapours from entering the pipette. Furthermore, the pipette should be stored suspended in a dedicated pipette stand. External contamination can be removed with soap solution or isopropanol. The individual parts should then be rinsed with distilled water and dried. Accidentally absorbed liquids should not be allowed to dry; the piston must be cleaned, and a small amount of silicone grease must then be applied.

Regular inspection of pipette condition

The precise and correct dispensing of samples and reagents is of prime importance for both research and diagnostic applications. To ensure reliable results, it is necessary to check the dispensing devices used for this purpose for proper function at regular intervals. Guidelines stipulate the regular control of pipettes and dispensers as well as the tools used for inspection.

To check for leaks, the nominal volume of the pipette is aspirated into the pipette tip (distilled degassed water) while the pipette is held vertically. The pipette, pipette tip and test liquid should all have the same temperature.

A visual check for leaks, broken parts, air bubbles and contamination should be carried out on a daily basis.

Preparing laboratory solutions

Preparing a solution is an important laboratory skill and must always be performed carefully and precisely. Although in IVF laboratories, the majority of solutions are commercially sourced at the appropriate concentration, a clinic embryologist still requires a basic understanding of solution chemistry and how to perform dilutions and produce buffer solutions.

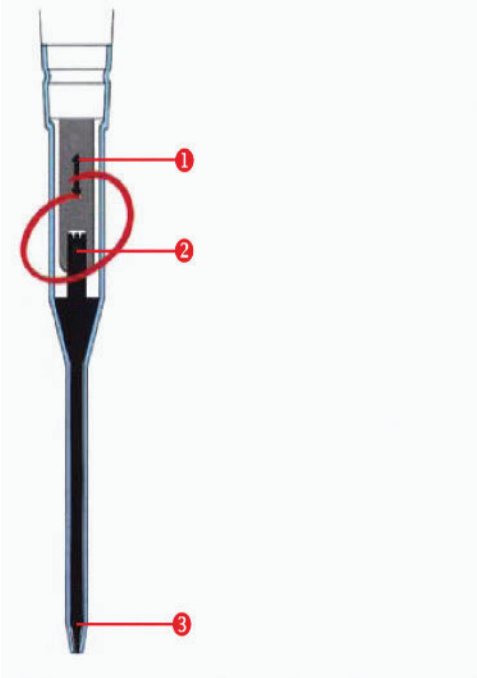

Figure 23. 6 Coupling of a positive displacement pipette with a matching pipette tip: the tip has an integrated piston (2) which is securely connected during pipetting with the piston rod of the pipette (1). The liquid in the tip only reaches up to the hermetic sealing lip (3), thus the formation of aerosols is excluded. Figure reproduced with permission from Eppendorf UK.

A solution is defined as a homogeneous mixture of two or more substances; frequently (but not necessarily) a liquid solution. Of significance to the preparation of solutions (and discussed here) are the dissolution of solutes in a solvent (e.g. water) forming a homogenous liquid, how solutes affect the properties of the solution, electrolytic dissociation, molecular dissolution resulting in charged particles, osmotic effects and the effect of solute particles reducing the effective concentration of the solvent and biological effects.

Solubility is the property of a solute to be dissolved in a given amount of solvent at a given temperature to form a homogeneous solution of the solute in the solvent. A solution is said to be unsaturated as long as more solute can be dissolved.

- *Dilute or weak solutions*: Contain small amounts of solute compared to the solvent.
- *Concentrated solutions*: Contain large amounts of solute to solvent, but still below the maximum.
- *Saturated solution*: The concentration of the solute is the maximum possible under the given conditions of temperature and pressure. A solution

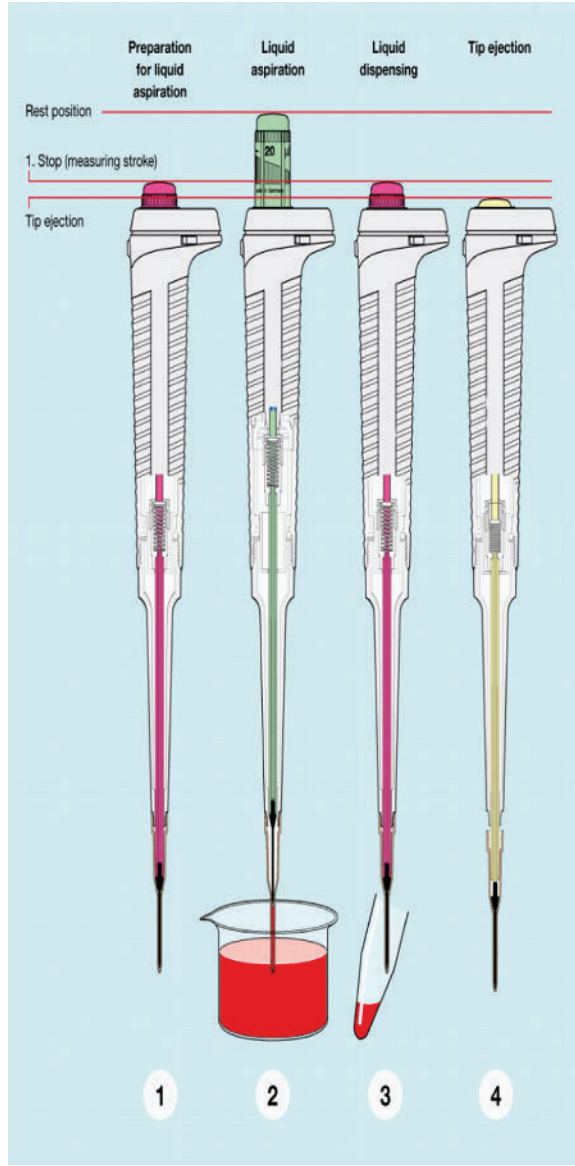

Figure 23.7 Function of a positive displacement pipette. To prepare for aspiration of the liquid (1), the push-button is pressed to the first stop and the piston moves down to the corresponding position. For aspiration of the liquid (2), the pipette tip is immersed a few millimetres into the liquid vertically, the push-button is then allowed to slide back slowly, the piston moves up, and the required volume of liquid is aspirated into the tip by the partial vacuum that is produced. To dispense the liquid into a vessel (3), the push-button is slowly pressed to the first stop. The piston in the tip is moved down by the piston rod of the pipette, thus displacing the liquid from the tip. The push-button is held down, and the tip drawn up against the wall of the vessel. To eject the tip (4), the push-button is pressed all the way down. Figure reproduced with permission from Eppendorf UK.

is said to be saturated when no more solute can be dissolved at the given temperature and pressure. A saturated solution is one in which the dissolved and undissolved solutes are in equilibrium.

- *Supersaturated solution*: Contains more dissolved substance than a saturated solution; the solution is not in equilibrium with the pure substance and the supersaturation is maintained by the application of energy (as, for example, temperature or pressure). An example of supersaturated solutions is carbonated water.

When a solute is added to a solvent, the properties of the solution are changed with respect to those of the unadulterated solvent. Specifically, the solution will have a lower vapour pressure (less volatile), higher boiling point and lower melting point. The osmotic pressure will also be changed. These effects depend only on the number of solute particles in the solvent.

Solution concentration

The concentration of a solution is equal to the amount of compound dissolved (the solute) in a certain volume of solvent and is measured in *Molar Concentration*, which is the number of moles of solute per litre volume of solution (mol l^{-1}), more commonly given as *molar* (M), and is a measure of the amount of substance per unit volume of solution (concentration = amount/volume).

Molar values and concentration

A mole is a unit of measurement for an amount of substance, has the unit *mol* and is defined as the amount of substance that contains as many elementary entities (e.g. atoms, molecules, ions, electrons) as there are atoms in 0.012 kg of the isotope carbon-12 (^{12}C)[1]. Thus, by definition, one mole of pure ^{12}C has a mass of exactly 12 g.

Molarity is a measure of the concentration of a solution. Molarity (M) = moles of solute (mol) / solution volume (litres). Thus, a 1 molar solution contains 1 mole of solute in 1 litre of solvent. For example, the mass of 1 mole is given by the Relative Molecular Mass (RMM), which is 58.44g for NaCl. Therefore, a one molar solution of NaCl contains 58.44g of NaCl in 1 litre of solvent. However, volume is temperature dependent (*n.b.* pV = nRT, where p = pressure, V = volume, n = no. of moles, R = 8.314 (Gas Constant) and T = temperature), so molarity can change with temperature. Therefore, we can express the concentration of solute relative to the mass of solvent (mol kg^{-1}), so that molality becomes the number of moles of solute (mol) / mass of solvent (kg). This is a temperature-independent way of expressing solute concentration, although this is rarely used unless the osmotic properties of a solution are of interest [3].

Molarity – worked example

Calculate the molarity of a solution prepared by dissolving 22.0g of sodium chloride in enough water to make a total of 225mL of solution. Express your result as mol l^{-1}.

Calculate the number of moles of solute

Number of moles of solute = Mass of solute / Mass of a mole of solute

RMM sodium hydroxide (NaCl) = 22.9898g (Na) + 35.453 (Cl) = 58.4428g

Therefore, no. moles = 22g/58.4428g = 0.376 moles

Here, molarity is requested in mol l^{-1}. As we have less than 1l of water, we must therefore calculate how many moles of NaCl we would have in 1l.

Calculate concentration per litre

We have 0.376mol/225ml.

In 1l of solvent, we have 1000ml, and therefore can contain our solvent volume (225ml) 1000/225ml = 4.44 times. Therefore, in 1l, our no. solute moles = 0.376 x 4.44 = 1.669mol. Thus our concentration is 1.669mol l^{-1}. Note that this is the equivalent of dissolving 97.54g of NaCl (1.669mol x RMM 58.4428g) in 1l of solvent. 97.54g/4.44 = 22g and thus we can check to ensure our calculations are correct.

Concentrations expressed as % w/w

This notation is commonly seen in laboratories, but often misinterpreted. % w/w is defined as percent weight per weight. In other words, this represents the solute mass in g per 100g of solution, so that a 5% w/w sucrose solution represents 5g sucrose and 95g water. Here we assume a density of water at 1 g ml^{-1} giving a total solution mass of 100g of which 5g is solute (5g/100g × 100/1 = 5%).

Concentrations expressed as % w/v

Here, the definition of notation implies percent weight per volume. This represents the number of grams of solute per 100ml solution, so that 5% w/v sucrose solution represents 5g of sucrose in 100ml solution.

Concentrations expressed as % v/v

This notation implies percent volume per volume and represents the volume of liquid solute per 100ml of solution. Thus, 5% ethylene glycol v/v solution represents 5ml of ethylene glycol in 100ml solution. Again, the % does not refer to an actual percentage but is a legacy notation. This is particularly useful for solutes whose relative molecular mass isn't known, e.g. cellular proteins.

Non-SI (Système International) Units

The International System of Units, normally abbreviated to SI from the French *Système international d'unités*, is the modern form of the metric system and is the most widely applied system of measurement in science. Utilization of standard measurements systems is important in science (and commerce) as it allows researchers to directly compare results performed using different techniques and methodologies. For example, parts per million (ppm) literally is the number of solute units per million solvent units and is equivalent to 10^{-6}g per ml, or µg ml^{-1}. Thus a 1.0ppm solution has a concentration of 1.0µg ml^{-1}. Parts per billion (ppb) is similar except that ppb is equivalent to 10^{-9}g per ml, or ng ml^{-1}.

PPM – worked example

ppm = mass of A in solution x 10^6 / total mass of solution

How to prepare a solution

Use a container twice as large as the volume of the solution you wish to make and if using water as a solvent, use distilled or deionized water. Define the concentration of solution and the volume of solution required (i.e. determine what concentration and how much you require). Add the measured volume of solvent (water) to the container. The solvent should be continuously stirred by magnetic stirrer (if the volume of water is measured using a volumetric flask, the stirring flea should be added after the volume is finalized). Calculate the relative molecular mass of the solute from available chemical data sheets or from the sum of the atomic masses of component elements. Calculate the molar amount of solute required and then, using the molecular mass, the mass of solute required. While continually stirring, add the desired amount of solute.

How to create a solution – worked example to prepare 250ml of 0.1 mol l^{-1} NaOH

Calculate the RMM of NaOH

RMM NaOH = 22.9898g (Na) + 15.9994 (O) + 1.00794 (H) = 39.99714g

Volume solvent required

250ml = 0.25 litres

Mass solute required

Concentration solution = No. moles solute/volume solvent

0.1 mol l^{-1}= no. moles solute / 0.25l

No. moles solute = 0.1mol l^{-1} x 0.25l = 0.025mol

Multiply no. moles by RMM to determine the mass of solute required:

0.025mol x 39.99714g = 0.9999285 g NaCl.

So to give 0.1 mol l^{-1} NaCl, add 0.9999285g of NaCl to 250ml water.

Weighing out the solute mass

When weighing out the appropriate mass, if the weighed mass is too large then consider making up a larger volume. Using the process above, the new volume may be calculated from the mass of solute available. Alternatively, you can make up a stock solution which can be subsequently diluted (see Serial dilution, below). If the chemical sticks to the weighing boat, wash off the remnant solute into the mixing vessel. For accurate measurements, rinse the original vessel with water and use this to make up volume. It is often useful to dissolve the solute in slightly less then total volume, stirring and heating as necessary. If heat is used to aid solute dissolution, the pH should be checked once the solution is cool (if necessary) and the solution then made up to the correct volume.

Serial dilution

Serial dilution refers to the process of reducing the solute concentration of a solution through the addition of solvent. For example, a one-in-ten dilution has 1 volume of original solution with 9 equivalent volumes of solvent. The additional solvent is referred to as the diluent (as it dilutes the solution). This may be represented as a ratio, 1:10, showing the initial and final volumes, or as 1:9, showing the volume of stock and diluents. The dilution factor is used to calculate the volumes of stock and diluent needed and is defined as the ratio of the initial concentration of the stock and

the final concentration of the diluted solution and may be calculated by dividing the required concentration by the concentration of the stock solution (see below).

Creating a serial dilution

When performing a serial dilution, ensure the solution is thoroughly mixed (undissolved solute will make the dilution inaccurate) and ensure you use a fresh pipette or tip for each step as very dilute concentrations can easily be compromised by residue, higher concentrations, remaining in used tips.

Creating a serial dilution – worked example

Calculate dilution factor

To prepare 100ml of NaOH at 0.2 mol l^{-1} from a stock solution of 10.0 mol l^{-1}:

Dilution factor = Required concentration / Starting concentration = 0.2 / 10.0 = 0.02

Dilution factor = 0.02 (or fiftyfold dilution from 1 ÷ 0.02)

Calculate volume of stock solution

Volume of stock solution = Volume required x dilution factor = 0.02 x 100ml = 2ml

Then the remaining volume of 100ml is made up using diluent (100 – 2 = 98ml).

Linear dilution series

Dilution series are used to create a series of solutions whose concentrations are separated by an equal amount. We can use a simple equation to determine the amount of stock solution required for each dilution:

$$[C_1]V_1=[C_2]V_2$$

where C_1 is the initial concentration, V_1 the initial volume, C_2 the new concentration and V_2 the new volume. A step interval is chosen so that the concentration interval is that required. Using this equation allows us to cover a broad range of concentrations.

Doubling dilutions

In a doubling dilution series, each concentration is half that of the first ($\log_2$ dilution series). In each case, an equal volume of solution and diluent is chosen and the dilution repeated identically as required. From *doubling dilutions* we obtain dilutions that have *dilution factors* of two-, four-, eight-, sixteenfold, etc.

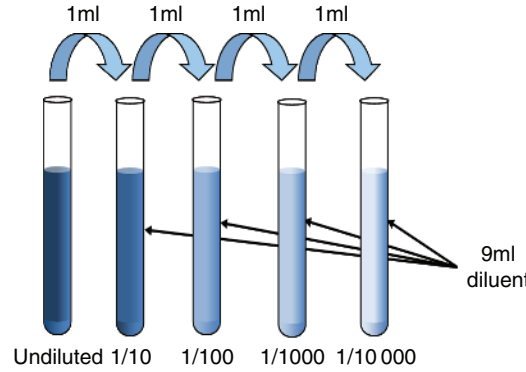

Figure 23.8 Example of decimal dilution whereby 1ml is sequentially transferred into 9ml of diluent.

Decimal dilutions

For decimal dilutions, each concentration is one-tenth of the previous one (i.e. $\log_{10}$ dilution series) and is achieved by the stepwise transfer of a constant volume to which is added a constant volume of diluent (see Fig. 23.8).

Stepwise (reciprocal) dilution

Here, the dilution series follows a pattern of the reciprocals of successive integers, i.e. 1/1, 1/2, 1/3, 1/4, 1/5 etc. and is achieved by a stepwise increase in diluent volume. For example start with 1, 2, 3, 4 and 5 times the volume of diluent to which is added a constant volume of stock. This method will prevent the dilution transfer errors that can occur with other dilution methodologies, although it will not produce a linear dilution series as the step interval is not linear.

Intermediate mixtures

Intermediate mixtures are those that are neither homogeneous nor heterogeneous, for example, when one component is dispersed evenly through another. These intermediate mixtures are commonly termed colloids and consist of mixed particles larger than molecules and/or ions which are too small to separate from the dispersion medium with gravity. Intermediate mixtures therefore consist of two separate phases: the dispersed, internal phase and the continuous, dispersion medium phase. Common examples include milk and gelatine. Colloidal suspensions can scatter rays of light, a process known as the Tyndall effect, and they may also be polar with a hydrophobic and a hydrophilic end.

Polar colloids can act as emulsifiers of fats and oils in aqueous solutions.

Serial dilution – worked example

If we require a final antibody in solution concentration of 2µg/ml in a final volume of 500µl from a stock antibody solution of concentration of 1 mg/ml, we must

convert mg/ml to µg/ml

2 mg/ml = 2000µg/ml

Convert µl to ml

500µl = 0.5ml

Therefore, for 2µg/ml in 0.5ml (500µl) we require (2/1000 mg) x 0.5 = 0.001ml of stock solution, made up to 0.5ml with solvent.

Microscopy in clinical embryology

Microscopy is a fundamental technique in ART, aimed to identify, monitor and score gametes and embryos at various stages of their development. For the precise manipulation and detailed visualization of gametes and embryos, embryologists use different types of optical systems such as stereomicroscopes, compound and inverted microscopes, equipped with a wide range of illumination systems. Understanding the optical principles of the different microscope systems and the type of illumination used is therefore an integral part of an embryologist's training.

This section aims to introduce the basic optical principles of the different types of microscopes used in ART and the reason for selecting a specific type to carry out different ART procedures as well as to provide information on their general maintenance to ensure accurate analysis and consistency.

Microscopes integral to an ART unit

Choosing a microscope for visualizing live cells without compromising viability is one of the major considerations in ART laboratories. While light intensity and exposure time are not issues for stained and fixed samples, they must be limited when working with living cells.

It is inevitable that gametes and embryos are observed outside of the incubator's controlled environment, and even under culture oil, it is almost impossible to avoid fluctuation of temperature inside the media drop containing gametes and embryos. It is well know that even small changes in temperature can cause irreversible damage to the integrity of the meiotic spindle in oocytes and have a negative impact on the developmental potential of embryos [4–6]. Therefore it is essential that the microscope used is equipped with a heated stage to maintain the optimal 37°C in the culture drops to reduce temperature fluctuation. The optical system used also needs to be able to generate high-resolution images for accurate and quick analysis to limit time spent outside the incubator. Another important consideration for successful imaging of gametes and embryos is that currently all staining methods used to highlight specific cell components are toxic to live cells. To overcome this issue, specialized illumination techniques such as phase contrast and Hoffman modulation contrast are utilized to achieve high-contrast imaging of the otherwise highly translucent gametes and embryos.

To meet all the above considerations, ART laboratories are routinely equipped with several different types of optical systems. These most commonly include stereomicroscopes, upright and inverted compound light microscopes, each of which is specifically selected to carry out different ART procedures depending on their magnification power, basic setup options and their ability to generate high-contrast images [7].

Optical principles of compound light microscopes

Compound light microscopes are one of the most commonly used basic optical systems. These are called compound microscopes as they use more than one lens to magnify the image (objective and ocular lens) and are light microscopes as they use visible light to illuminate the sample (Figure 23.9). In order to achieve high-quality imaging it is recommended to use the Köhler illumination system, which aims to generate even illumination of the sample via a system of diaphragms to avoid visual artefacts from the light source and minimize heat transmission. The light generated by the light source is transmitted through the Köhler illumination system in the base of the microscope to the condenser lens that focuses the light onto the sample. As light passes through the specimen, it is collected by the objective, one of the most important elements of the microscope that determines magnification and resolution of the generated image. The objective is selected using the revolving nosepiece, and the image is passed on to the ocular lens in the eyepiece, which projects the final image onto the retina of the observer. The total magnification potential of a microscope is given by the

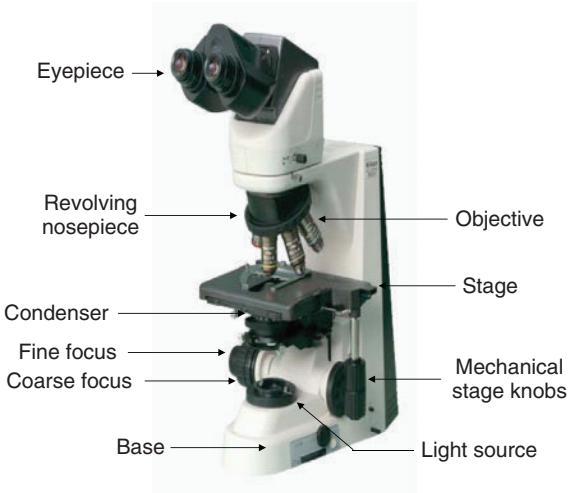

Figure 23.9 Components of a compound microscope. Figure reproduced with permission from Nikon Instruments UK.

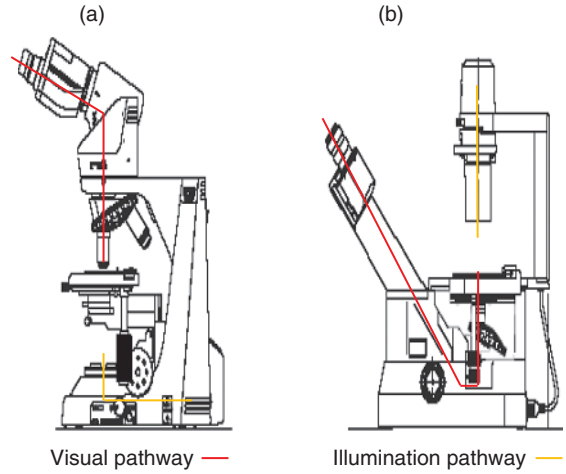

Figure 23.10 Optical principles of (a) upright and (b) inverted compound microscopes. Figure reproduced with permission from Nikon Instruments UK.

magnification power of the objective multiplied by the magnification power of the eyepiece. For instance, using a 10× objective, the image is magnified 100× with a standard 10× ocular lens.

One of the most commonly used compound microscopes is the upright microscope, where the condenser directs the light onto the sample from below and the objective collects the light reflected from the sample above the microscope stage (Fig. 23.10a). In this setup, the sample is observed on a glass slide covered with a cover slip and the working distance between the objective and the cover slip is small. Inverted compound microscopes, on the other hand, illuminate the sample from above and view the object from an inverted position, i.e. the objective is found below the sample (Fig. 23.10b). This type of setup enables examination of living cells in their 'artificial' culture conditions in a petri dish and allows more space for the use of micromanipulation tools.

Optical principles of stereomicroscopes

Stereomicroscopes, also referred to as dissecting microscopes, are designed for low-magnification observation of samples. They are essentially composed of two compound microscope optical trains running parallel to each other and a sheared large objective. This is the most routinely used design, referred to as the Common Main Objective (CMO) design (Fig. 23.11). As the light passes through the large main objective it is transmitted through the parallel right and left channels containing zoom lenses and an image inverting prism system to transmit the image through the eyepieces to the observer (Fig. 23.11).

Unlike compound microscopes, which generate a two-dimensional high resolution image, stereomicroscope systems result in a three-dimensional image with high contrast but with a limited resolution and magnification power. Higher quality stereomicroscopes are also equipped with a zoom lens system to enable rapid changes in magnification. One of the main advantages of stereomicroscopes is that they offer a larger field diameter and have a much longer working distance (20–140 mm) compared to compound microscopes, which enable the safe and accurate manipulation of living cells and tissues. On the other hand, for the same reasons stereomicroscopes are not the best optical systems to carry out detailed observations at the intercellular level.

Microscope illumination systems

The type of illumination chosen to visualize unstained live samples is an important aspect to generate high-contrast detailed images. Contrast is the difference in light intensity between different parts of the sample compared to their surrounding and the background. While the degree of resolution and magnification are of crucial importance to achieve good quality images, unstained live samples are highly transparent, and without contrast-enhancing techniques, details of the sample would not be visible to the observer.

The most common illumination systems used to assess gametes and embryos are described below, each

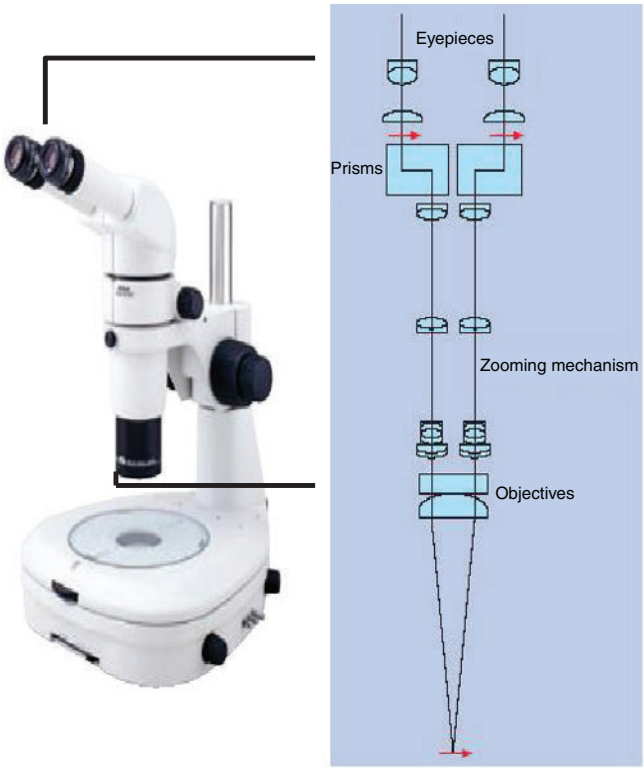

Figure 23.11 Components and optical principles of stereomicroscopes (CMO). Figure reproduced with permission from Nikon Instruments UK.

of which are aimed to enhance contrast at various degrees to visualize intercellular details for accurate clinical analysis.

Brightfield illumination

Brightfield illumination is the default method used in light microscopy. The sample is evenly illuminated resulting in a bright background, while any differences in the reflectivity or light absorption of the specimen's surface result in reduction of light reflected back to the objective, causing that part of the image to appear darker than the background. This means that under brightfield illumination, detailed visualization is dependent on the light absorption properties of the sample. This type of illumination is most suitable for the observation of naturally pigmented or stained and fixed samples as it provides very little or no contrast to unstained live material.

Darkfield illumination

Darkfield illumination is a specialized contrast-enhancing illumination system that highlights details of the sample against a dark background. This is achieved by using an opaque disc placed in front of the condenser to shield direct light and illuminate the sample with diffracted light waves. Light reflected from even surfaces will not reach the objective (dark background), while some of the diffracted light scattered by the uneven surface of the sample passes through the objective giving a brighter appearance to that part of the image. This system is very easy to use and cost efficient, and it can be applied to any light microscope. Its main disadvantage however is the low contrast levels, requiring a much higher light intensity that can be damaging to live samples.

Phase contrast illumination

Phase contrast illumination is a technique that aims to give contrast to otherwise invisible or highly translucent samples. This can be achieved by the addition of a condenser annulus plate placed in the front focal plane of the condenser and a matching phase plate placed in the rear focal plane of the objective (Fig. 23.12). The sample is illuminated by a defocused hollow cone of

light using the annular plate. Depending on the density and reflective index of the sample, the light either passes through the sample unchanged (direct light) or is retarded to various degrees (diffracted light). The phase plate is aimed to selectively slow down the direct light by one-fourth of a wavelength. This amplified difference in phase between direct and diffracted light results in a contrast-enhancing effect within the sample that is visible to the observer. Phase contrast is a relatively cost-efficient method to produce high-contrast, detailed images of samples with low light absorption properties that would otherwise be invisible under brightfield illumination. The generated image however is two-dimensional with a low depth of focus. Phase images are prone to optical artefacts that appear as bright or dark hollows surrounding the outline of details that can distort the final image. In addition, the phase plate limits the numerical aperture of the objective which in turn results in diminished resolution.

Hoffman modulation contrast

Hoffman modulation contrast (HMC) emphasizes the different gradients of the sample using a special filter called the Modulator, a slit polarizer and an HMC objective (Fig. 23.13). The modulator placed in the back focal plane of the objective is constructed to have three distinct regions, each with different densities. The dark zone transmits 1% of light; the middle grey zone transmits 15%, while the bright zone transmits 100% of light. The HMC system generates a phase gradient within the sample using a condenser slit plate placed in the front focal plane of the condenser, which is aligned with the grey zone. As light passes through different gradients within the sample, light is refracted onto the different areas of the modulator, resulting in difference in contrast and the generation of a three-dimensional grey-scale image. This technique offers an excellent resolution of details and generates high-contrast images. In addition, 'optical sectioning' allows observation of each plane of the specimen without interference. This means that the entire depth of the specimen can be viewed in sections in excellent resolution. Most important, HMC can be used to view live samples in plastic or glass petri dishes without distorting the image or generating optical artefacts.

Nomarski illumination system

Nomarski illumination, also referred to as Differential Interference Contrast (DIC) microscopy, is another method used to generate high-contrast images of transparent living material. DIC is a beam-splitting interference system that uses two Nomarski prisms (modified Wollaston prisms). Transmitted light generated from the light source first passes through a polarizer, which transmits the light onto the first Nomarski prism underneath the condenser, which splits the light into two sheared

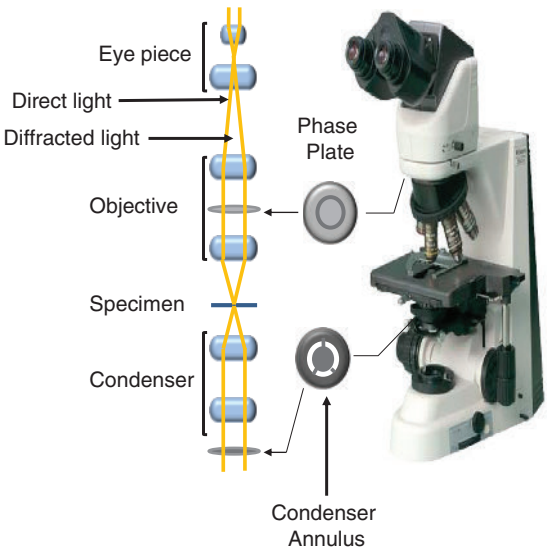

Figure 23.12 Optical principle of phase contrast microscopy. Figure reproduced with permission from Nikon Instruments UK.

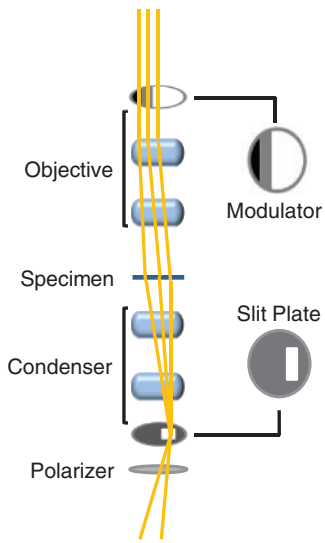

Figure 23.13 Hoffman Modulation Contrast system with detailed modulator and slit design.

light waves (Fig. 23.14). The two light beams pass through the condenser and the specimen, where their path is altered depending on the reflective index and density of the specimen. These differentially modified light waves are collected by the objective and are transmitted through the second Nomarski prism at the rear focal plane of the objective and the analyzer, where they are recombined. The combined light wave passes through the eyepiece to the observer, generating a shadow-effect image with different light intensity and colour at varied areas of the specimen.

Unlike the HMC system, however, the high-contrast, three-dimensional like image generated by this method does not show the real geometrical surface of the specimen. Also, it is not suitable for analyzing tissue culture specimens, as the plastic tissue culture dish interferes with the optical system, resulting in misleading images.

The choice of optical systems in ART applications

When selecting a microscope to carry out various techniques in the ART laboratory, one needs to consider the properties of the sample to be analyzed (e.g. spermatozoa are much smaller in size (40 µm in length) compared to oocytes (110–115µm), the level of magnification and resolution required and whether the technique involves detailed observation or manipulation of gametes and embryos. Based on these considerations and the above discussed advantages and disadvantages of the commonly used optical systems, it is a rather logical choice as to which microscopic technique is the most suitable for the various ART procedures (summarized in Fig. 23.15).

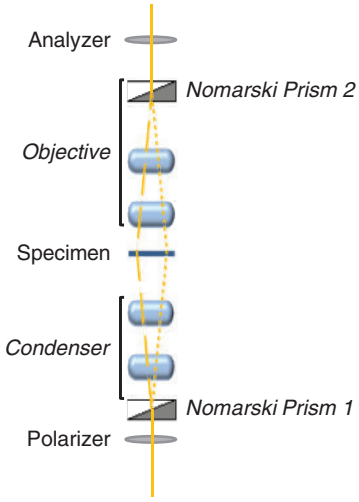

Figure 23.14 Optical principle of Differential Interference Contrast imaging using Nomarski Prism optical system.

Manipulation of oocytes and embryos

ART applications, such as oocyte collection, transfer of oocytes and embryos between culture dishes, embryo thawing and vitrification, involve precise and accurate manipulation under the microscope. To carry out these techniques, brightfield stereomicroscopes are the preferred choice, as the low-magnification, three-dimensional image generated allows embryologists to orient themselves under the microscope and provide enough space for safe movements of oocytes and embryos within/between dishes [7]. Due to the large field of view and long working distance, embryologists can quickly scan through dishes containing follicular fluid to identify cumulus-oocyte complexes (COCs). Using the zoom function, the maturity of granulosa

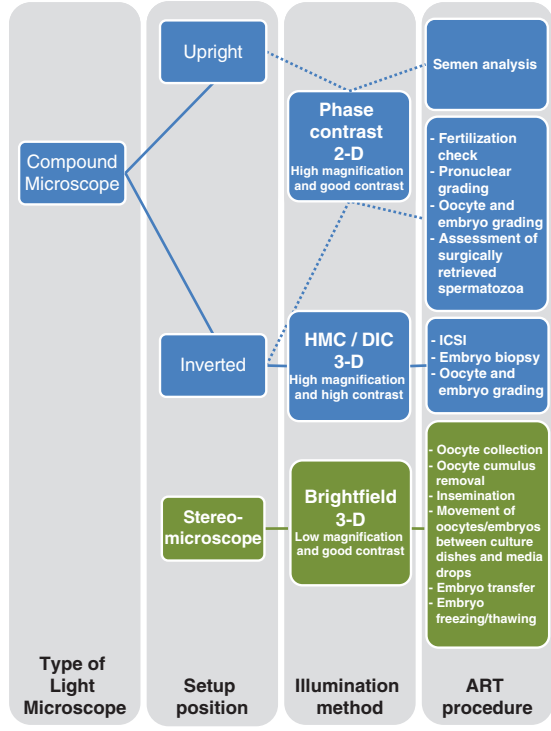

Figure 23.15 Summary of the different types of optical systems used in ART.

cells as indicators of oocyte maturity can be determined as well as confirming the presence of polar body following removal of corona cells in preparation for intracytoplasmic sperm injection (ICSI).

Semen analysis

While the accurate manipulation of oocytes and embryos is the major consideration when choosing stereomicroscopes, semen analysis involves a more detailed morphological and structural analysis of spermatozoa. Semen analysis routinely involves the evaluation of sperm count, motility and morphology at the microscopic level, which is carried out using an upright compound microscope equipped with phase contrast optics [7]. Phase contrast is used to visualize the otherwise highly transparent spermatozoa against their background and enable accurate analysis. The high magnification power (100×–400×) at high resolution is required to achieve a detailed morphological assessment of different parts of the sperm cell (head, neck, mid-piece and tail), a detailed investigation to overcome some of the main causes of male factor infertility [8, 9].

Grading of oocytes and embryos

While analysis of spermatozoa on a flat glass slide using an upright compound microscope is suitable for detailed observation, grading of oocytes and embryos requires the use of an inverted microscope equipped with phase contrast illumination [7]. This 'inverted' setup allows imaging of oocytes and embryos in their optimal culture condition as there is enough space to place a petri dish onto the microscope stage. The high magnification and resolution of this optical system enables detailed observation at the intracellular level, which involves grading of pronuclear morphology, number and size of blastomeres, presence of multinucleation, degree of cytoplasmic fragmentation and detailed grading of blastocyst trophectoderm cells and inner cell mass. The phase contrast optical system is used to give contrast and visualize these key markers as indicators of embryo viability and developmental potential.

Micromanipulation of gametes and embryos

Micromanipulation of gametes and embryos such as ICSI and embryo biopsy involves the precise coordination of micromanipulation tools in order to achieve fertilization or to obtain a small sample for PGD or PGS analysis, respectively. The success of these techniques is dependent on the embryologist's skills but also on the quality of the optical system. For both procedures, embryologists require excellent visualizing systems that can generate three-dimensional, highly detailed images. ICSI requires a clear image of the oocyte in order to orient the polar body precisely at the 12- or 6 o'clock position and ensure that the sperm is deposited in the middle of the cytoplasm. Embryo biopsy, however, requires excellent image resolution and contrast for accurate positioning of the laser beam for zona pellucida drilling and the removal of a single blastomere, or a small part of the trophectoderm, to minimize damage to the embryo as much as possible.

These micromanipulation procedures entail the use of a high-quality inverted microscope and optical system, most commonly HMC, to generate a high-contrast, three-dimensional image of the sample at high magnification (100–400×) [7]. The Nomarski illumination system is also a good option; however this is less popular as it requires a special injection or biopsy dish to avoid optical interference of plastic and glass dishes.

Advances and novel microscopy techniques in ART

For more than a decade, non-invasive imaging with light microscopy has been in use for the detailed morphological and structural analysis of gametes and embryos. While this method does help to identify morphologically abnormal gametes and embryos, it cannot provide enough information to differentiate between embryos of the same grade. In order to identify novel non-invasive markers, technological advances in the field of microscopy are aimed at visualizing gametes and embryos in even greater detail.

For instance, the combination of high power magnification (6000×) and a contrast-enhancing technique (Nomarski DIC) has facilitated the highly detailed observation of spermatozoa for intracytoplasmic morphologically selected sperm injection (IMSI), a modified ICSI technique. Observation of spermatozoa using high magnification microscopy allows grading at the organelle level, such as nuclear morphology, presence of nuclear vacuoles and midpiece

morphology. All of these non-invasive markers have been shown to increase fertilization and pregnancy rate compared to traditional ICSI procedure [10–12]. Although this technique is highly time-consuming, it is the perfect example to show that visualization of live spermatozoa using advanced optical systems can reveal unseen intracellular details that are useful markers to increase IVF success rate.

Another good example is the use of polarized light microscopy (PLM), a specialized contrast-enhancing technique that is used to visualize birefringent (double-refracting) components of live cells. Polarized illumination requires the use of a polarizer and an analyzer (second polarizer) within the optical pathway to modify the basic optics of a light microscope. Accumulating evidence suggests that visualization of birefringent components of gametes and embryos, such as the meiotic spindle, layers of the zona pellucida and spermatozoa acrosome, are useful prognostic markers in ART. One of the main potential uses of PLM is to determine the presence and location of the meiotic spindle to avoid damage to the genetic material during ICSI. Several studies have shown that the presence of spindle has a positive prognostic value for fertilization rate and embryo developmental potential [13–15]. In addition, due to the highly sensitive nature of the spindle to external factors such as temperature and pH, its presence or absence can be used to optimize culture conditions [16, 17].

Under polarized light, the multilayered architecture of the zona pellucida is also visible and the intensity of birefringence from the inner zona layer has been found to correlate with embryo developmental potential and pregnancy [18–20]. PLM has also been found to aid in selecting spermatozoa for ICSI by visualizing the acrosome. Selecting competent acrosome-reacted spermatozoa, which show birefringence only at the post-acrosome area compared to the whole sperm head in non-reacted spermatozoa, Gianaroli and colleagues have found that this non-invasive marker is a valuable predictor of pregnancy outcome after ICSI when treating severe cases of oligoasthenoteratozoospermia and selecting sperm from testicular biopsy samples [21, 22].

While current microscopic observation of embryo development is only possible at snapshot intervals and requires the exposure of embryos to unfavourable conditions outside of the incubator, the ultimate goal would be to generate spatial-temporal imaging of the developing embryo within the incubator. The EmbryoScope system, which enables time-lapse imaging of embryos in their culture conditions, represents a new generation technique to gain more information about embryo development. Having an overview of the history of each embryo's development can potentially identify novel prognostic markers, such as the time of first meiotic division and synchronized cell division [23].

Maintenance of microscopes

Microscopes represent a significant investment and are essential instruments in an ART laboratory. They require regular mechanical and optical maintenance to ensure consistent image quality, which can be affected by contaminants such as dust, grease and dirt. These contaminants can be airborne or originate from the user, especially in areas where hands, eyelashes and moisture from breathing are in direct contact with the instrument. Buildup of dust, smudges on the optical elements of the microscope as well as scratches on the lenses can significantly diminish optical performance. While it is recommended that full maintenance of microscopes be carried out by specialized personnel annually, regular basic maintenance is recommended, especially for heavily used instruments. This may include the systematic cleaning and inspection of the mechanical and optical components of the microscope, as described below.

Basic cleaning of mechanical components

Mechanical components, such as the microscope stage, focusing knobs and control rods, are in direct contact with the observer and are exposed to skin oil and moisture. Therefore it is essential that these areas are decontaminated regularly with a moistened lint-free cloth or alcohol wipes and dried with a clean cloth. The painted surfaces of the microscope may also be wiped and decontaminated regularly.

Basic cleaning of optical components

It is essential that lens surfaces are cleaned with appropriate lens cleaning products, as conventional cleaning solvents may cause irreversible damage to the delicate optical components of the microscope. As a routine

recommendation, loose particles from the eyepiece lenses, objective lens and the condenser should be blown off using an air blower. If required, lint-free lens paper or a cotton swab applicator can be used to remove any contaminant that cannot be removed with the air blower. To remove greasy smudges or immersion oil, lens cleaning solutions may be used as recommended by the manufacturer. Preventive action to reduce accumulation of airborne particles may include the use of microscope covers when the instrument is not in use.

Summary

Within this chapter we have attempted to describe some of the fundamental skills required by a clinical embryologist. Of course, many of these skills are also applicable to multidisciplinary laboratories. We hope to have provided sufficient information for young embryologists to establish a basic framework with which to develop their practical laboratory skills. However, it is important to note that this chapter does not describe all of the key points one needs to consider before working in a laboratory, especially a clinical laboratory. It is important to note that each laboratory will have its own set of key rules, and that infrastructure and equipment inventory may vary significantly. Furthermore, while the basic skills required in a laboratory do not change significantly over time, equipment style and operation do change dramatically, as can experimental protocols. Moreover, health and safety guidance regarding recommendations for handling chemicals and equipment are in a constant state of flux, and significant consideration should be afforded to such legislation.

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Chapter 24

Semen analysis and preparation

Aysha Itani

Introduction

This chapter describes general semen analysis in detail and discusses the various methods of semen preparation techniques that are currently being used in assisted reproductive laboratories (ART) today.

Male factor infertility contributes to approximately one-third of all assisted reproduction referrals [1]. It is therefore essential when investigating couples presenting for fertility treatment that an accurate analysis of their semen is undertaken.

The new edition of the WHO laboratory manual for examination and processing human semen (WHO Manual) was released in 2010. This should be considered the gold standard for all semen analysis and preparation methods and describes them all in detail. Many ART laboratories still use the 1999 reference manual, although changes arising from the 2010 update are now beginning to phase in. Both sets of reference standards will be referred to in this chapter, with the newer values clearly indicated in italics [2, 3]. Recent modifications to the WHO manual were essential, as earlier versions did not take into account the time to become pregnant (fecundity) when defining the normal semen characteristics of a fertile man [4].

Semen analysis

There are four main stages of semen analysis:

1. Patient history
2. Sample production
3. Semen analysis
4. Interpretation of results

Patient history

It is important to determine relevant patient history when analyzing semen, as sometimes very obvious factors can have significant implications on results, for example, illness with fever, medication, past medical and surgical history and occupational and leisure activities. There are many everyday events that can affect the quality of sperm undergoing analysis, and some of these are discussed in Table 24.1.

Sample production

Ideally, sample production rooms should be located as close to the laboratory as possible so that samples can be analyzed as soon after liquefaction as possible. At the Oxford Fertility Unit, hatches adjoin the laboratory so the sample can be handed directly to the laboratory after collection. The semen sample should be procured into a sterile single use container that has been clearly labelled with the patient's full name and original identifier. Washed containers should never be used as they may contain soap or residue from its previous contents. The sample should be produced by masturbation and the time taken noted. Whether the whole sample was collected or not should also be noted on an accompanying slip. If the first part of the ejaculate is missed it may influence analysis as the latter part is less spermatozoa rich [3]. Some men often find it difficult to produce on their own, and on demand, and may need their partner to help them. If condoms are to be used then silastic condoms without spermicide must be utilized in order to avoid the toxic effect of latex and spermicide which would obviously be harmful to any sperm found within.

The average time for production is 10–15 minutes [13]. If the patient takes significantly longer than this, then clinical decisions regarding the cryopreservation of samples for use on the day of treatment should be considered. This will both alleviate the stress upon the patient of producing a sample on the day, and ensure that a useable sample is available when required.

In order to accurately assess and reproduce semen analysis results, samples should be produced only after

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

Table 24.1 Everyday events and their fertility implications

	Event	Fertility implication
Reproductive history	Previous pregnancies	Male has been fertile in the past
Abstinence	2–7 days	Longer periods of abstinence may reduce sperm motility, shorter periods may reduce total motile concentration and volume [5]
Diet	Poor diet vs. well-balanced diet	Good diet enhances sperm quality [6]
Caffeine	Consuming increased amounts	Increased levels of caffeine can negatively affect semen quality [7]
Alcohol	Excessive alcohol consumption	Decreases sperm motility [8]
Smoking	Smoking cigarettes	Decreases semen quality [8, 9]
Radiation/ chemical exposure	Prior to chemotherapy or occupation related	Deleterious affects on semen quality [10]
Driving	Prolonged sitting	Warms testicles and consequently decreases optimal sperm production [11]
Medicine/past surgery	Vasectomy, congenital bilateral absence of vas deferens (CBAVD) Steroids	No sperm should be present in vasectomy and antisperm antibodies may be present if reversed. CBAVD should result in azoospermia. Use of steroids can show marked decrease in spermatogenesis [12]

2–7 days of abstinence. Shorter periods of abstinence may result in a reduction in both semen volume and sperm concentration. Conversely, increased periods of abstinence may result in a higher percentage of non-moving sperm. If additional samples are required, then periods of abstinence should be consistent, in order to permit comparable results [5].

Semen analysis

It is essential that at least two samples are analyzed in order to gain a suitable assessment of the patient's specific semen characteristics. Individual parameters tend to be variable and in order to ascertain the characteristics of an average sample, it is best to collate the results of more than one sample. The normal standards as defined in the WHO manual are listed in Table 24.2.

Basic semen analysis is made up of four components: volume, sperm motility, count and morphology. As soon as the sample is produced it should be placed in a warmed non-CO_2 incubator to accelerate liquefaction. Liquefaction refers to the natural change in semen consistency over time from a gel-like vesicular secretion to a liquid [3, 5]. A non-liquefied sample cannot be analyzed accurately as sperm are unable to move freely. Typical liquefaction time is 20 minutes but liquefaction can occur almost immediately after ejaculation. Delayed liquefaction of semen, more than 1 hour after ejaculation, may indicate disorders of accessory gland function, or infection.

Semen analysis should take place as soon as liquefaction has occurred, ideally 30 minutes after production and certainly before 60 minutes have elapsed, in order to prevent the sample from evaporating and deteriorating within the seminal plasma. The sample should be removed from the incubator and inspected by looking at the sample through the pot. This can establish sample presence and permit assessment of general consistency. Due to the heterogeneous nature of semen, the sample should be mixed well by hand rotation prior to analysis. Samples should not be vortex-mixed as this will be detrimental to the sperm within.

Normal semen samples may contain gelatinous bodies. While these can affect the production of wet preparation slides, they do not appear to have any clinical significance. The presence of mucus streaks, which may also be seen during initial analysis, can interfere with motility parameters [3]. Normal semen is a homogenous grey-opalescent colour and variations in this colour may be indicative of disease. If a sample is oligospermic then it is less opaque. A red/brown colour indicates the presence of red blood cells. This could be indicative of infection and should always be discussed with the patient and clinician to ascertain that no serious conditions are evident. Semen can also have a yellow appearance due to jaundice or some vitamin supplements [3].

Table 24.2. WHO reference values

Parameter	WHO 1999 [2]	WHO 2010 [3]
Volume	> 2ml	1.5ml
Appearance	Grey and opaque	Grey and opaque
pH	> 7.2	> 7.2
Concentration	> 20M/ml	> 15M/ml
Motility	> 50% progressively motile	> 32% progressively motile
Morphology	> 30% normal	> 4% normal
Leucocytes	< 1M/ml	< 1M/ml

Volume and viscosity

The first part of semen analysis is to determine volume and viscosity. At the Oxford Fertility Unit, volume is calculated using a 5 ml or 10 ml graduated pipette. The normal volume for semen is between 2 and 5 mL (*1.5 ml*). Low semen volume, together with an azoospermic sample, i.e. a sample containing no sperm, can be characteristic of ejaculatory duct obstruction or congenital bilateral absence of the vas deferens. If the volume is too high, this may indicate increased abstinence or inflammation of the accessory glands, especially if the pH is low [3].

The WHO manual recommends calculation of volume by measuring the weight of the container containing the sample. By using a container of known weight, the weight of the sample can be extrapolated and the precise amount of functional spermatozoa can be calculated as there will be no remnants in the pot or pipette. Volume loss can be in the order of 0.3 ml and 0.9 ml [3].

While measuring volume, it is customary to measure the pH of the sample using pH paper with a range 6.0–10.0. pH should also be analyzed within 1 hour of production as pH is affected by the loss of CO_2 that occurs following ejaculation and will therefore increase with time.

Viscosity is then graded by allowing the sample to gravitate into its container and analyzing the stream. At the Oxford Fertility Unit viscosity is graded into four categories:

1. Reduced, sample appears watery
2. Normal, leaves the pipette in small, discrete drops
3. Slightly increased – can be loaded into a pipette tip easily
4. Greatly increased – does not load into a pipette tip easily (in this case cutting the end of the tip will facilitate the sample)

Abnormally increased viscosity is defined as when the thread from the gravitating semen is more than 2 cm long [3]. Increased viscosity does not appear to have any clinical significance; however, it does reduce initial movement parameters and consequently this reduces the capacity of sperm to penetrate the cervical mucus in vivo. After preparation, and the removal of functional sperm from seminal plasma, normal movement parameters are returned [14].

Sperm motility

Two × 10 µl separate aliquots should be placed on a clean glass slide and covered with a 22 mm by 22 mm cover slip. This 'wet prep' can then be analyzed under a phase contrast microscope for presence of mucus streaks, agglutination, presence of infection by increased numbers of round cells and most importantly the presence of spermatozoa. The required magnification is dependent on what is being observed, but to detect the presence of bacteria, a minimum of 400× is recommended [3]. Sperm motility can be calculated using this slide at a power of 200× magnification or 400× depending on concentration. Using a higher power will facilitate the analysis of concentrated samples.

The calculation of motility of ejaculated sperm is an extremely important characteristic that has a high association with fertility [15]. It can be calculated using computer-aided sperm analysis (CASA), but in the clinical setting it is considered far more accurate to determine motility manually using a cell counter and a phase contrast microscope.

In order to calculate motility, sperm are separated into four categories:

(a) Progressive motility > 20μm/s at 20°C
(b) Slow or sluggish
(c) Non-progressive motility < 5μm/s
(d) Immotile

To perform motility analysis, an area must be defined within the field of view (see Fig. 24.1). This area is systematically scanned and all sperm of category 'a' and 'b' are scored and then the field is returned to score and calculate the categories 'c' and 'd'. To achieve an acceptably low counting error, at least 200 sperm should be counted with a laboratory counter [3]. The sperm counted should be spread over at least five fields of view. These values are then expressed as a percentage. This should be repeated on a second aliquot and the results compared. If there is less than 10% difference between the highest categories on the two aliquots then this is deemed acceptable; if not, then a third slide should be prepared and analyzed and the results averaged. If it is not possible to count 200 sperm, this should be noted and the numbers not expressed as percentages.

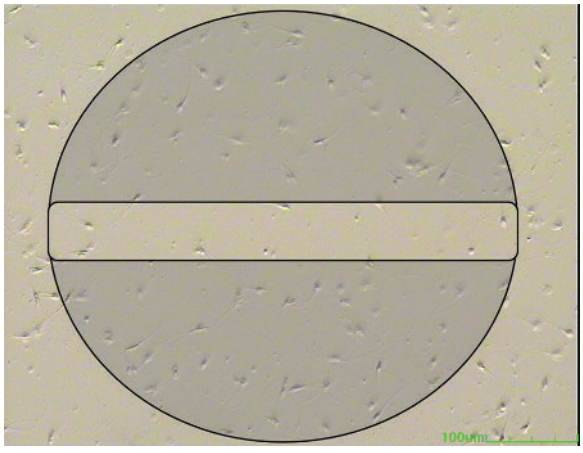

Figure 24.1 Field of View. Lightened area is systematically scanned for progressively motile and slow or sluggish sperm and then return to field to calculate non-progressive and immotile sperm.

The WHO 2010 has simplified the calculation of motility by grading sperm into three categories. (a) Progressively motile, these sperm are actively moving and going either in a straight line or in a circle at any speed, i.e. previous a and b. (b) Non-progressive motility, this includes all other types of motile sperm, i.e. shaking, twitching or moving in very small circles, and (c) immotile sperm, the lack of any movement at all.

It should be noted that the velocity of sperm is temperature dependent and can double when assessed at body temperature as opposed to room temperature [16].

Concentration

The determination of an accurate concentration is important due to its association with fertilization rates and time to conception [5]. A count is considered normal if the concentration of sperm is greater than 20M/ml (*15M/ml*). The concentration of sperm can be calculated in a variety of manners. The most accurate and reproducible method of counting sperm is through dilution to immobilize the sperm and to use a haemocytometer. Due to the viscous nature of sperm it is important to use a positive displacement pipette so as to minimize errors in the dilution phase. A variety of dilutions can be used, but if a 1 in 20 dilution is used then the number of sperm in five large boxes of a Neubauer Haemocytometer is the equivalent of the number of sperm per ml (see Fig. 24.2). However, it is critical not to overcount the number of sperm; therefore only whole sperm should be counted (those with heads and tails). In order not to count the sperm twice, only sperm with the majority of its head in the square should be counted and even then, only if it is on one of the two boundary lines that have been allocated to count. This will prevent overestimation.

There are other methods for counting sperm, and these include the Makler chamber and Horwell

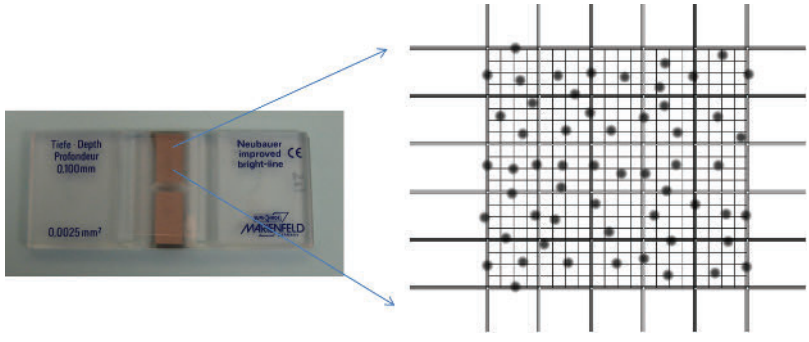

Figure 24.2 Haemocytometer and representation of grid within. Indicating 25 large squares and 16 smaller ones, the cells should only be counted when the majority of sperm is within the square.

Fertility Counting chamber, both of which have a ten by ten counting grid and use undiluted semen. The advantage of this method, i.e. the ease and speed of use, is counteracted by a substantial error rate as care must be taken not to overfill these chambers. When overfilled, the chambers do not allow for a single layer of sperm to be created and thus introduce a source of error. While these techniques may be quite usual for a busy, non-specialized laboratory, they should not take the place of the method recommended by the WHO manual for accurate analysis [17].

Sperm morphology

Sperm morphology is important in the evaluation of a semen sample; however, there is no clear boundary between fertility and infertility with normal sperm. If there are grossly abnormal morphological defects, then the fertilizing capacity is greatly diminished. The variable morphology of human sperm makes the analysis quite difficult and there are various common classification systems with the more recent editions of the WHO manual quoting the Kruger method and the more strict Tygerberg Criteria.

A normal, human sperm has a smooth, regular oval shape, with a well-defined acrosome area that takes up approximately 40–70% of the head area. The head should be whole and contain no vacuoles. Normal sperm are approximately 4.1 microns in length with a median width of 2.8 microns [3] (see Fig. 24.3). The midpiece should be 0.6 microns in width, regular and its length should be in the order of approximately 4 microns.

The tail, or principal piece, should be uniform along its length and thinner than the midpiece. It should be unbroken and have no kinks or coils and be approximately ten times the size of the head, i.e. 45 microns long. Various abnormalities of head, neck, midpiece and tails exist. Full and concise descriptions are available in the WHO manual [3].

Analyses involve both stained and unstained methods. There are many commercially available kits available to stain sperm. It is possible to perform simple morphological assessment upon raw semen. However, while such practice will not be accurate for diagnostic laboratories, it may be sufficient in a clinical setting. If there is an increase of grossly abnormal sperm, then ICSI (intracytoplasmic sperm injection) may be recommended. This is especially true for globozoospermic samples where the acrosome is missing and conventional fertilization would not normally be possible.

Commercially available kits assist with morphological assessment by differentiating between various components of the individual sperm (head, acrosome, midpiece and tail) by staining, e.g. Diff-Quik, TestSimplets (MidAtlantic Diagnostics, Inc, Mount Laurel, NJ) or SpermMac (Autosperm, Fertipro, Lotenhulle, Belgium). Slides are prepared in accordance with the manufacturer's instructions. These are then scored according to normality or not. Staining methods are particularly useful as the sperm are immobilized and clearly identifiable.

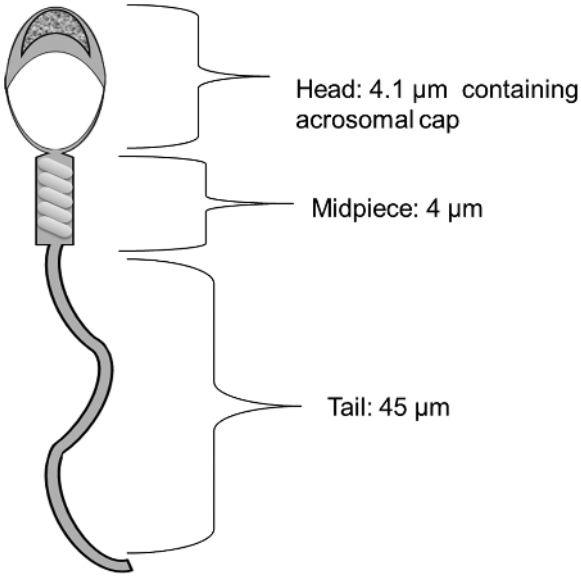

Figure 24.3 Normal sperm and median lengths

Other aspects of analysis

In addition to concentration, count and morphology, there are further investigations that can be performed in order to make a complete analysis of semen quality.

Anti-sperm Antibodies (ASABs)

Mature sperm are produced after puberty and because of this they are sometimes recognized as foreign proteins by the immune system. While the sperm are within the testicle they are protected by tight junctions of the Sertoli cells. When there is a breach of this 'blood-testis' barrier, an immune response may occur. The most common reasons for this are vasectomy, testicular biopsy and testicular injury, e.g. torsion and infection [18]. Antibodies are then produced and secreted into the prostatic and seminal fluids.

These antibodies may cause sperm to agglutinate, preventing forward progression and thus diminishing their fertilizing capacity, or become cytotoxic and kill sperm. Some antibodies prevent sperm-cervical mucus interaction or prevent zonal binding. If there is an increase in ASABs, then it is of clinical advantage to use ICSI in order to reduce the risk of failed fertilization that could be associated with poor sperm motility or binding [19].

The Mixed Agglutination Reaction (MAR) test is one of the many ways to test for antisperm antibodies. This test is performed by mixing a small aliquot of semen with latex beads that are coated with immunoglobins. If antibodies are present then the motile sperm form clumps with the beads; if not, then the sperm will continue to swim freely about the slide [19]. However, most laboratories now use the Immunobead test as this can determine which antibodies (IgA, IgG and IgM) are present. In this test, semen is combined with latex beads coated with IgA or IgG and incubated. If antibodies are present then the beads attach directly to the sperm. This test provides more information than other tests as the beads can attach to the head, neck, midpiece or tail and thus determine the specific location of the ASABs [20].

Sperm vitality

The vitality of sperm is normally tested by assessing the ability of the plasma membrane to prevent the introduction of certain dyes or stains. Sperm vitality is not routinely analyzed in andrology laboratories. However, such methods can be useful in identifying rare cases of necrospermia, as opposed to total sperm motility, e.g. Kartagener Syndrome [4].

The Hypo-Osmotic Swelling Test (HOST) is another means of identifying sperm vitality. This test is based on the ability of live sperm to withstand moderate hypotonic conditions by measuring the ability of the sperm plasma membrane to transport water. Dead sperm, whose membranes are no longer intact, do not exhibit swelling and thus can not function during the fertilization process. Live sperm will show controlled swelling and their tails will curl. When the sperm are placed back in normal media, the tails should recover and they can be used for ICSI [21].

Reactive oxygen species

The generation of Reactive Oxygen Species (ROS), or free radicals, is normally associated with the infiltration of leucocytes that are present in semen. ROS have been associated with roles in capacitation and cell signalling pathways when present at low levels [22]. However, ROS can also be generated in excess when certain samples are prepared on density gradients. Spermatozoa within these samples have the capacity to create additional ROS. This is a sign of impaired sperm maturation or abnormal morphology and has been associated with male factor infertility. These sperm retain excessive levels of residual spermatid cytoplasm in the midpiece and are also associated with reduced fertilizing ability [23].

ROS are metabolites of oxygen and include hydrogen peroxide, nitric oxide, superoxide anion, along with hydroxyl and hydroperoxyl radicals. This is critical for the andrology laboratory because when ROS are present at high levels they can cause pathological cellular damage. Oxidative damage to cellular lipids, proteins and DNA can occur. Although most cells have anti-oxidant defence mechanisms, when these fail, sperm function can be impaired.

DNA fragmentation

Human sperm chromatin is highly condensed, but single and double helix breaks can occur over time. Normally these are repaired by integral mechanisms; however, at some stage, this damage becomes non-repairable and can be transmitted to any resulting embryos [24].

A high level of DNA fragmentation within human spermatozoa may represent a cause of male factor infertility that is overlooked by normal semen analysis. The fertilizing capacity of sperm with increased DNA fragmentation is not always affected; however, increased genetic abnormalities will have significant consequences on embryo development to blastocyst stage. There is also a poor prognosis for a successful pregnancy due to an associated increase in miscarriage rates [24].

In order to measure the levels of DNA fragmentation in a sample there are many tests available, e.g. TUNEL, COMET or commercially available kits such as Halosperm [Halotech Dna, Sl, Madrid] that uses the Sperm Chromatin Displacement test. In these tests, semen is tested for the levels of DNA fragmentation by immersing sperm into an inert gel. The cells are treated in order to denature the DNA in those exhibiting high levels of fragmentation. The nuclear proteins are washed away and the slide stained. Sperm with minimal or no DNA fragmentation will show large halos, whereas those with increased fragmentation may not show a dispersion halo at all.

Table 24.3 Summary of results and treatment options

Condition	Description	Treatment option
Normozoospermia	Production of sperm in normal numbers and motility	IUI or conventional IVF
Oligozoospermia	Low sperm count. Concentration < 20 Mill/ml	ICSI Backup (Either conventional IVF or ICSI depending on severity on day of clinical treatment)
Asthenozoospermia	Reduced sperm motility. Progressive motility < 50%	ICSI if severe
Teratozoospermia	Reduced numbers of sperm with a normal appearance (morphology). Normal morphology < 30%	ICSI if severe
Cryptozoospermia	Apparent azoospermic sample but where sperm are found during analysis only after centrifugation	ICSI – In these cases it may be best practice to freeze a sample prior to treatment to ensure availability of sperm on day of treatment
Necrozoospermia	Samples with only dead sperm (not necessarily just immotile sperm)	Treatment with their own sperm is not presently an option
Globozoospermia	Often referred to as 'round-head' defect. Sperm morphological defect where the acrosome is absent and sperm usually have small round heads	ICSI
Aspermia	No ejaculate, i.e. complete lack of semen, e.g. retrograde ejaculation	ICSI if sperm is found
Azoospermia	No spermatozoa in the ejaculate obstructive: where sperm are created, but cannot mix with the rest of the ejaculatory fluid due to a physical obstruction (e.g. CBAVD) non-obstructive: where there is a problem with spermatogenesis	If sperm found then ICSI

Interpretation of results

Once semen analysis has been completed, a recommendation for treatment protocol can then be made and options considered (see Table 24.3).

Sperm preparation

Seminal fluid contains a wide range of factors other than spermatozoa that must be removed in order to optimize the number of sperm available for fertilization. Vitality and motility diminish in time. This is due to the presence of decapacitation factors within the seminal plasma. It is therefore essential that samples are prepared as soon as possible after production in order to remove spermatozoa from the seminal plasma and its harmful effects. The presence of even a small amount of seminal plasma within the preparation can prevent capacitation so it is important that a clean preparation is produced in order to concentrate the optimal sperm for fertilization [25].

While preparing semen, damage to sperm must be minimized by avoiding large fluctuations in temperature and unnecessary centrifugation. The centrifugal forces used while separating motile sperm from the seminal plasma should be the lowest possible in order to maximize sperm yield and minimize the production of ROS.

Preparation methods

There are four main preparation methods:

1. Dilution and washing
2. Sperm migration
3. Density gradient
4. Commercially available products

Whichever method is utilized, all plasticware, glass and media should be checked for toxicity to sperm and embryos, with a sperm survival test, prior to first use.

The choice of preparation method will generally vary between laboratories but essentially it will depend on the sample itself. At the Oxford Fertility Unit, most samples are prepared on a dual density gradient. However, a severe oligoasthenospermic sample is best prepared on a 40% gradient as this will condense the sample and the viable sperm will usually swim out in long drops. A long drop is an elongated drop of media under oil where the preparation is placed. The drops are allowed to settle and are then examined.

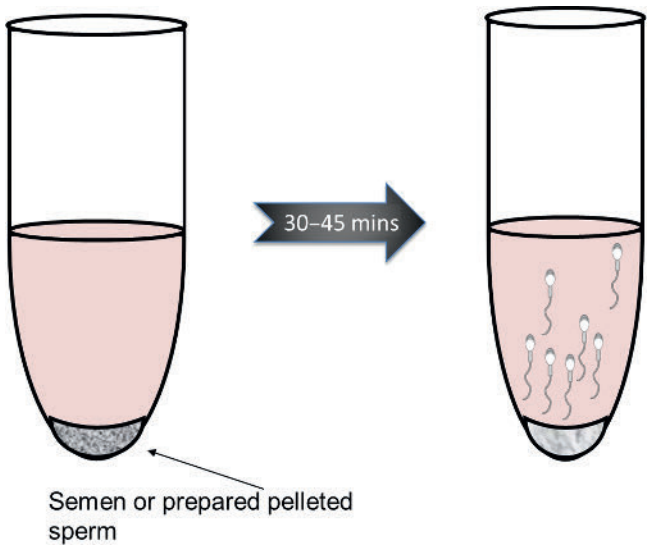

Figure 24.4 Sperm migration. Semen is placed under media and the motile sperm allowed to swim up with time.

Sperm, if present, can then be individually picked up using the injection pipette if required for ART.

Dilution and washing

Raw semen is diluted with a large volume of culture media and separated. The advantage of this technique is that it is a simple procedure that washes away the seminal plasma. The disadvantage is that all sperm, including dead sperm, are pelleted, thus decreasing the fertilizing capability by impairing the functional motility of the good sperm. It also does not reduce ROS and should only be used with ICSI [4].

Sperm migration (swim up)

Here, functional sperm are separated by migration. Small aliquots of liquefied semen are placed under a layer of culture media and left to swim out. This is usually done by carefully using a syringe and a needle filled with semen. It is important to ensure that there are no air bubbles in the syringe and that the semen is expelled slowly. Once the allocated amount has been placed under media, the needle is sharply pulled upwards, avoiding drawing any semen up. It is possible to create the swim up by layering culture media over the semen; however, care must be taken not to dislodge the lower layer. Multiple tubes can be used in order to maximize sample yield. The tubes should be left at an angle in order to increase semen/media interface (see Fig. 24.4). The samples are left so that the motile portion of sperm move up into media and then after 30–45 minutes, depending on the quality of the sample, the supernatant is retrieved and centrifuged at 500*g* (0.5 rcf) for 5 minutes in order to concentrate the sample. A motility and concentration analysis should then be performed in order to calculate the number of motile sperm present. The whole process should be performed at 37°C [17].

There are many advantages of a swim up, the biggest being that it creates a highly motile preparation. This method is beneficial for use on samples that have an increased amount of cellular debris or round cells as these do not clog up the gradients. It is also a suitable technique to use for samples that do not respond very well to the centrifugation method and remains cost effective as it does not require expensive gradients or chemicals other than the culture media.

However, the disadvantages of this method are that it is not suitable for every sample. Very viscous samples respond poorly to this technique as the semen tends to pull up into the media and thus 'contaminate' the spermatozoa/media solution. Prolonged exposure of sperm to the seminal plasma means that samples with significant levels of ASABs are not suitable to be used with this technique, for, ideally, the sperm should be removed as quickly as possible from the semen [25]. Swim ups are also not overly suitable for oligospermic samples as they have notoriously low yields and thus insufficient sperm may be retrieved. The removal of the supernatant must be done very carefully in order to

avoid dislodging the seminal layer. It is best practice to remove little and often in order to maximize the success of the technique. It is also time consuming because of the incubation time.

Density gradient

The most common method of sperm preparation in the ART laboratory is selective washing. This utilizes density gradient centrifugation to fractionate subpopulations of sperm. During centrifugation the sperm reach a point in the density gradient that matches their own density. Motile sperm are separated from immotile sperm and seminal plasma by high-speed centrifugation through different gradients of commercially bought silane coated silica particles, e.g. Puresperm (Nidacon International AB, Gothenburg, Sweden). Normal sperm with more condensed DNA travels further to the bottom of tube during centrifugation. Round cells and abnormal forms with cytoplasmic droplets never make it to the pellet. However, overcentrifugation can cause ROS buildup if sufficient care is not taken. Care should also be taken to avoid overloading the gradient as this can 'block' the interface layer and prevent the passage of functional sperm [5, 17].

The advantages of density gradients are numerous; they are suitable for all samples including viscous samples and are a lot quicker than a swim up to perform. There is usually an increase in functional sperm that can be retrieved and hence a good overall yield and the gradients generally prevent the production of ROS [26]. However, there are disadvantages, for example, silicon particles are costly and not all samples respond well to the centrifugation technique. In these cases it is possible to perform a gradient followed by swim up in order to maximize the number of motile sperm available for use.

The density gradient is prepared in the following way. The lower, or more dense, layer is usually placed into the bottom of a sterile conical tube. This is usually 1–3ml 80% silica particles but can be 90% or 95%. The tube is then swirled to allow friction between the tube and the top meniscus to reduce. Carefully, the upper layer is added by swirling the solution slightly above the interface layer and allowing it to slide down the walls of the tube creating a clean interface. It is possible to underlay the heavier layer, but care must be taken not to blow bubbles which will compromise the interface. The semen is added on top, the tube closed and then centrifuged at 300*g* (0.3 rcf) for 20 minutes. Following centrifugation, the upper layer and interface should be carefully removed and then discarded. The pellet should be removed with a soft tip and then washed twice in order to remove any remaining silica particles (see Fig. 24.5). At the Oxford Fertility Unit, we use 2 × 5 min washes at *500g*. Following the final wash, the sample is resuspended in an appropriate volume depending on the technique to be used. For IVF a concentration of 5–10M/ml and for ICSI, 1–2M/ml is suitable.

Forty per cent centrifugation: this is a variation of the dual density technique described above and is used for removing seminal plasma from severe oligoasthenoteratozoospermic (OATS) or blood/tissue from

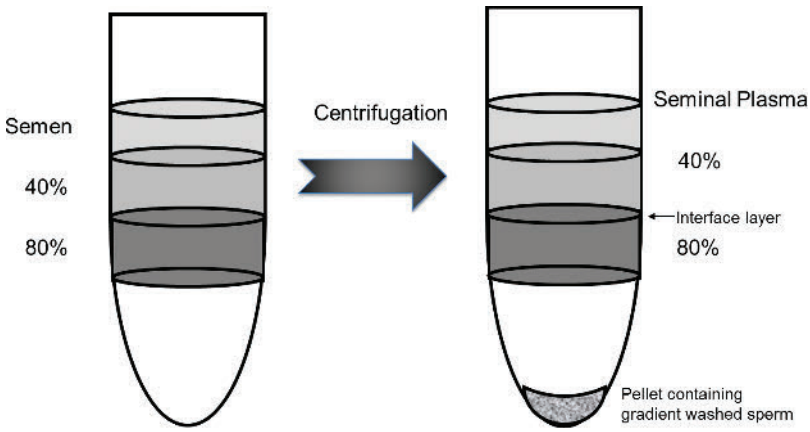

Figure 24.5 Density gradient. Following centrifugation, normal sperm are collected at the base of the tube in a pellet which should be removed and then washed to remove any remaining silica particles.

surgically retrieved sperm. This method concentrates samples to facilitate the recovery of sperm and should only be used for ICSI.

Commercially available products

There are many commercially available products to select sperm, most of which are based on the filtration column method. Motile sperm are separated from the non-motile fraction by a series of densely packed glass wool fibres. The principle behind this is based on the motility of sperm themselves and the ability of the glass wool to act as a filter as the sperm swim through the narrow fibres. The advantages are that these methods are simple to perform, there is usually a good yield and ROS are significantly reduced. However, these methods are expensive and the final preparation may not be as clean as the more conventional methods mentioned previously [17, 27].

Quality control and assurance

Within any laboratory it is essential to ensure that accurate analysis is undertaken. Most aspects of semen analysis are subjective and it is essential to ensure that strict standards are met within each laboratory. The UK NEQAS (United Kingdom National External Quality Assessment Schemes) in Andrology has a scheme whereby samples are distributed to participating laboratories for analysis (by DVD) for motility, concentration and morphology analysis (aliquot of formalin preserved semen). All participating laboratories are given the same samples and the results are correlated and analyzed centrally. This can be done both externally (by sending the results to the NEQAS coordinator who will analyze them) and internally (by getting all trained staff to analyze the samples and compare results). If any individual varies significantly in their analysis then retraining should be offered in order to maintain competency.

Witnessing

The Human Fertilisation and Embryology Authority (HFEA) requires that all ART units utilize strict witnessing protocols. This is to double-check the identification of all samples, and the patients or donors to whom they relate, at all critical points of the clinical laboratory process and to ensure no errors or 'mix-ups' occur. With some procedures this can be done manually with two members of staff checking and verifying; however, the majority of the checks can now be achieved electronically using a commercial product.

It is vital that any sperm preparation tubes are labelled clearly and it is good practice to only prepare one sample in a hood at any one time. The Oxford Fertility Unit uses electronic witnessing for its sperm preparation. Preparation tubes are electronically tagged, as is the sample production pot. These are both verified by a second physical witness at the start of the proceedings to ensure accuracy.

Summary

Semen analysis and preparation are important aspects of any assisted reproduction laboratory. Their results have significance whether in the clinical or diagnostic settings. It is therefore vital that these techniques are carried out accurately and consistently, following a training programme, with suitable quality control procedures in place to maintain standards.

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Chapter 25

Superovulation protocols

Janelle Luk and Pasquale Patrizio

Introduction

The ability to induce the development of one or multiple preovulatory follicles and attain mature oocyte(s) for pregnancy, in anovulatory and other infertility patients, remains one of the greatest achievements in reproductive medicine of the last 25 years. The main tenet of this process, known as ovulation induction, or controlled ovarian hyperstimulation (COH), is the administration of a particular set of medications known as fertility drugs, able to induce ovulation in anovulatory patients or to override the natural mechanisms of mono-ovulation. The growth of one or multiple follicles is then utilized for intrauterine inseminations (IUI) or in vitro fertilization (IVF). In this chapter, some of the common fertility medications used for ovulation induction, such as gonadotropins, agonists or antagonists of gonadotropin releasing hormone (GnRH), clomiphene citrate, and aromatase inhibitors are reviewed. Next, we discuss some of the common ovarian stimulation protocols used for IUI and IVF cycles. Finally, particular IVF protocols for patients with a specific ovulatory disorder (i.e. polycystic ovarian syndrome (PCOS)) or with different levels of ovarian response (i.e. poor responder vs. high responder) or with breast cancer (for fertility preservation) will be presented.

Indications

The patients with ovulatory disorders are classified by the World Health Organization in three groups [1]:

Group I – Hypogonadotropic hypogonadism – This classification includes women with hypothalamic-pituitary failure who typically exhibit low levels of follicular stimulating hormone (FSH), luteinizing hormone (LH) and estradiol. Patients with amenorrhea secondary to vigorous exercise or anorexia nervosa, or patients with defective hypothalamic-pituitary axis (as in Kallmann's syndrome), are representative examples of this group.

Group II – Hypothalamic pituitary dysfunction – This classification includes patients with PCOS and patients with anovulation either associated or not with hyperandrogenism, but not diagnosed as PCOS. Patients in this group usually present with normal FSH, LH and estradiol levels.

Group III – Ovarian failure (hypergonadotropic hypogonadism) – This classification includes patients with primary or secondary amenorrhea, with elevated FSH and LH and low estradiol levels indicating ovarian insufficiency or failure.

Physiology of follicular development

Ovarian folliculogenesis (approximately 85 days in duration) is regulated by both endocrine and intra-ovarian mechanisms that coordinate the processes of cell proliferation and differentiation [2]. There are two different phases of follicular development: (1) tonic growth phase or gonadotropin-independent, and (2) gonadotropin-dependent growth. The tonic growth phase involves the conversion of preantral follicles (primordial to primary and secondary follicles) with diameter of < 0.2 mm to antral follicles with a diameter up to 2–3 mm, which lasts for approximately 65 days [3]. The tonic growth phase is characterized by a 600-fold increase in granulosa cell numbers and a concurrent ~tenfold increase in diameter. Low circulating gonadotropin level support is necessary for follicular development.

The gonadotropin-dependent growth phase is an exponential follicular growth involving the conversion of antral follicles of 3–5 mm to a 20 mm preovulatory follicle and with concurrent 160-fold increase in granulosa cells endowment. The process of follicular recruitment encompasses the late luteal phase of the

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

preceding menstrual cycle, when estrogen levels fall following the involution of the corpus luteum and gonadotropin increase, and continues in the follicular phase up to cycle days 5–6. From this point on, the preselected follicles undergo growth, acquisition of dominance (in natural cycle) and final preovulatory maturation. In the early follicular phase, FSH must exceed a certain level to stimulate granulosa cell aromatase activity resulting in increased follicular concentrations of estrogen. The rising estrogen increases the follicular uptake of FSH and thereby supports the growth of the follicle in response to FSH action. During the early follicular phase of a natural, unstimulated cycle, the follicle that has produced relatively more estrogen becomes the dominant follicle. The lowering of circulating FSH levels caused by the estradiol produced by the dominant follicle promotes atresia in the rest of the cohort. In a stimulated cycle, the administration of gonadotropins during the early follicular phase causes synchronized growth in all the follicles, rescuing them from atresia and resulting in multiple preovulatory follicle development.

Ovulation induction medications

The agents commonly used for ovarian stimulation cycles are gonadotropins (FSH and LH), agonists or antagonists of GnRH, human chorionic gonadotropin (hCG) and other oral agents (Fig. 25.1). The choice of medications and protocols is highly dependent on the etiology of infertility and the type of ovulatory disorder. For example, patients with PCOS (WHO Group II) may first benefit from an oral regimen. Clomiphene citrate is the initial treatment of choice for most anovulatory or oligo-ovulatory infertile women who have normal levels of estradiol, thyroid stimulating hormone (TSH), prolactin and FSH levels. On the other hand, for clomiphene citrate–resistant anovulatory patients or patients with defective hypothalamic-pituitary axis (i.e. Kallmann's syndrome, WHO Group I), ovarian stimulation with exogenous gonadotropins will be required.

Medications

Gonadotropins

1. Human Chorionic Gonadotropin (hCG) extracted from urine of pregnant women or produced by recombinant technology
2. Human Menopausal Gonadotropin (hMG) extracted from urine of post-menopausal women (1 vial contains 75 IU of FSH and 75 IU of LH activity)
3. Follicle Stimulating Hormone (FSH) produced by recombinant technology (rFSH), or from urine after additional purification steps
4. Luteinizing Hormone (LH) produced by recombinant technology

Gonadotropin releasing hormone (GnRH) agonists/antagoinsts

1. GnRH agonists :
   - Leuprolide acetate
   - Nafarelin acetate
   - Buserelin acetate
   - Goserelin acetate
   - Triptorelin acetate
2. GnRH antagonists:
   - Cetrorelix acetate
   - Ganirelix acetate

Oral Regimens

- Clomiphene citrate
- Aromatase Inhibitors

Figure 25.1 A table with a list of medications that are used for ovulation induction.

Oral agents

Clomiphene citrate

Clomiphene citrate is one of the most commonly used oral agents for induction of ovulation. It is typically begun on the third or fifth day after the onset of a spontaneous or progestin-induced menstrual cycle. Patients usually start with a single (50 mg) tablet daily for a 5-day interval, and the dose can be increased, by 50 mg increments, in subsequent cycles until ovulation is achieved. Most women who respond to clomiphene do so at either the 50 mg (52%) or 100 mg (22%) dose level [4]. Even though not approved by the Food and Drug Administration, higher doses (150–250 mg daily) can sometimes succeed when lower doses fail [5]. Pregnancy rates are the highest during the first three cycles of clomiphene citrate treatment. During clomiphene citrate treatment, the levels of both LH and FSH rise, since clomiphene citrate, working as an estradiol antagonist, produces a negative feedback on the pituitary gland that favours the release of FSH and LH. Monitoring a clomiphene citrate cycle includes an ultrasound on cycle day 3 for baseline and on cycle day 11–12 for checking ovarian response. When the follicle size reaches 17 mm, hCG is given on that day. Intrauterine insemination will be performed 36 hours after hCG injection. It is interesting to note that clomiphene citrate is a pregnancy risk category X where studies in rats and mice have shown a dose-related

increase in some types of malformations and an increase in mortality. This is particularly important when considering the relatively long half-life of about 5 days to 3 weeks. However, studies in humans have not found an association between clomiphene citrate and congenital defects [6].

Some of the common side effects include transient hot flushes where 10% of treated women may experience such effect. Mood swings also are relatively common. Other mild and less common side effects include breast tenderness, pelvic pressure or pain and nausea. However, if neurological side effects like headaches or visual changes (i.e. blurred or double vision, scotomata and light sensitivity) arise, it is important to stop the clomiphene citrate treatment.

Letrozole

Another oral agent option for anovulatory or for clomiphene-resistant patients is letrozole, a drug that belongs to the class of aromatase inhibitor (another example is anastrozole). Although experience with its use is still limited, when used at doses of 2.5–5 mg, letrozole can decrease estradiol levels by 97–99% [7]. Letrozole is prescribed at the dosage of 2.5 to 5 mg daily on cycle days 3–7 to induce ovulation [7] and is completely absorbed after oral administration, with a mean half-life of approximately 45 hours (range: 30–60 hours). Clearance from the systemic circulation is mostly by the liver. Whereas clomiphene citrate stimulates endogenous FSH secretion by decreasing central estrogen negative feedback via estrogen receptor antagonism, aromatase inhibitors do so by inhibiting peripheral estrogen production which in turn triggers the release of pituitary FSH and LH. Aromatase inhibitors do not have direct anti-estrogenic effects on the endometrium as seen with clomiphene. In previous studies, endometrial proliferation was uniformly normal even though peak estrogen levels were 60–75% lower than was observed during previous clomiphene treatment. Teratogenic effects of letrozole have been suggested but not clearly proven in humans [8]. It is true that aromatase inhibitors are contraindicated during pregnancy. However, data indicate that fertility treatment with letrozole is safe and its use before conception does not seem to have increased risks for the fetus [9]. Some adverse effects include gastrointestinal disturbances, asthma, hot flashes, headache and back pain [10]. In summary, both clomiphene citrate and letrozole are oral agents that are generally used for ovulation induction starting on cycle day 3 to 7 for IUI cycles.

Injectables

Gonadotropins

Gonadotropins can be extracted from the urine of postmenopausal women or pregnant women (for hCG), or can be synthesized in vitro by using recombinant DNA technology (i.e. recombinant human FSH (rFSH), recombinant human LH (rLH) and recombinant human chorionic gonadotropin (rhCG)). The first gonadotropins introduced for COH were purified from the urine of postmenopausal women in the 1960s and labelled as human menopausal gonadotropin (hMG). Human menopausal gonadotropins are still available today with more refined extraction processing in order to create products with fewer urinary contaminants. Some contain both equal amounts of FSH and LH activity, while others contain primarily FSH with very low levels of LH or LH activity (i.e. given by the presence of hCG instead of LH) [11]. These products are available in vials as freeze-dried powder, which are reconstituted with diluent. Recombinant human FSH (rFSH) became available in the mid-1990s, and both recombinant human LH and recombinant hCG were also made available thereafter. These products are now available as multi-dose vials, prefilled syringes or pen devices. The benefits of recombinant medications include the elimination of contaminating urinary proteins, greater batch-to-batch consistency and precise bioactivity because the product is measured by the mass of the protein (i.e. 75 IU of FSH = 5.5 μg of protein) [12]. In ovulation induction cycles for intrauterine insemination (IUI), the usual starting dose of gonadotropin is 75 IU per day, increased to 150 IU if no ovarian response is achieved (Fig. 25.3). When using gonadotropins, it is highly recommended to provide close follicular monitoring and use the lowest starting dose to minimize the risk of multiple pregnancies and ovarian hyperstimulation.

GnRH agonists or antagonists

Prior to the widespread use of GnRH agonists, up to 35% of ART cycles were cancelled due to premature luteinization resulting from an unanticipated LH surge. The structure of native GnRH reveals a decapeptide structure with the sites responsible for the physiological actions: (a) activation of the GnRH receptor on the pituitary cells (amino acids [aa] 1–3); (b) regulation of GnRH receptor affinity (aa 5–6); and

(c) regulation of biologic activity (aa 9–10) [13]. These three areas of the molecule can be modified to change the properties of the native molecule to produce GnRH analogues. The GnRH analogues are classified as agonist or antagonist. The agonists first produce an acute release of stored pituitary gonadotropins known as the 'flare' effect. This causes a rise in endogenous LH and FSH and in turn a rise in estradiol level. After approximately 10–14 days of continuous GnRH analogue administration, there is a loss of receptors from the surface of the pituitary gonadotropes, while the remaining receptors will become less sensitive, a process known as desensitization or down regulation. This results in a decline of FSH, LH and in turn ovarian estradiol levels. The GnRH agonists commonly used are leuprolide acetate, nafarelin acetate, buserelin acetate, goserelin and triptorelin.

The GnRH antagonists are structurally very similar to the GnRH receptor. They exert a competitive binding with the native receptor, thus causing a prompt decline of FSH and LH levels without the flare effect of the GnRH agonist. GnRH antagonists act within a few hours after their administration [14]. This is in contrast to GnRH agonists where pituitary down-regulation occurs after some 10–14 days. The two GnRH antagonists available for clinical use are ganirelix and cetrorelix, both showing the same biopotency and effectiveness. For ganirelix acetate there are many more substitutions with unnatural amino acids as opposed to the GnRH analogues at positions 1, 2, 3, 6, 8 and 10. Substitutions at positions 1, 2 and 3 are important for the antagonistic effects. The substitution at position 6 helps protect against enzymatic degradation and enhances aqueous solubility [13], while substitutions at positions 8 and 10 help to reduce the release of histamine that plagued earlier generations of GnRH antagonists.

Human chorionic gonadotropin (hCG)

The hCG serves as a substitute for the LH surge, necessary for the final stages of oocyte maturation with the completion of Prophase I to Metaphase II of meiosis and the detachment of the cumulus oocytes complex from the follicular wall [15]. The hCG can be either urinary-derived or synthesized by recombinant technology. Urinary hCG is used at a dose of 5 000 to 10 000 IU, while recombinant hCG is dosed as 250 micrograms which is equivalent to approximately 6750 IU of hCG. Within 35–36 hours of hCG administration, the oocytes are retrieved from the follicles using transvaginal ultrasound guided aspiration [15, 16].

Factors Affecting Choice of Protocol

- The age of the woman in addition to her baseline FSH, Anti-mullerian Hormone (AMH) and E2 levels
- Antral follicle count
- Body Weight
- The etiology of infertility
- The patient's history of prior stimulation outcome (normal response vs. poor or hyper-response)

Figure 25.2 A table with a list of factors that may affect the choice of protocol for each particular patient.

Protocols for IVF

The choice of stimulation protocol varies according to the cause of infertility, age of the patient, body mass index (BMI), ovarian reserve and response to previous cycles of assisted reproduction or ovulation induction (see Fig. 25.2). In general, the ovarian stimulation protocols can be divided into two groups: (1) long or luteal phase protocols and (2) short or follicular phase protocols.

The long protocol

In a typical long protocol, the administration of GnRH agonist begins in the midluteal phase of the previous cycle, approximately 1 week after ovulation, at a time when endogenous gonadotropin levels are at or near their nadir (see Fig. 25.3). For leuprolide acetate, the usual dosage begins with 0.5 mg subcutaneous daily until the onset of menses and it is then decreased to 0.25 mg when gonadotropin stimulation starts (and continued until the day of hCG administration). For nafarelin acetate, the initial dose is 400 μg twice daily as nasal spray and decreased to 200 μg when stimulation starts. At the onset of menses, the GnRH agonist-induced suppression of serum estradiol levels (less than 50 pg/ml) and the absence of ovarian follicular activity (i.e. no ovarian cyst (s)) need to be established before the initiation of gonadotropin stimulation. The dose of the gonadotropins (rFSH alone or together with rLH or hMG; or hMG alone) is kept constant for the first 4–5 days of stimulation. For patients with normal prescreening results, demonstrating normal ovarian function, and for patients who are younger than 38 years old, the usual starting dosage is between 150–225 IU. The patient's initial response is then assessed by transvaginal ultrasound (to visualize the number and size of developing follicles) and serum estradiol level on cycle day 5. If the follicular

recruitment proceeds slowly with estradiol level < 150 pg/ml, the gonadotropin dosage can be increased, or LH/hMG can be added if not already in use. On the other hand, if the follicular response is rapid and the estradiol level is higher than 500 pg/ml or few follicles are already of advanced size (15 mm or greater), the dose of gonadotropin can be decreased. The majority of the programmes adopt the step-down regimen, characterized by a reduction in the daily dose of gonadotropins as ovarian response progresses in the late follicular phase. The hCG is administered when an appropriate number of follicles have reached a mean diameter between 18–20 mm.

The short protocols

The two common short protocols are (a) the micro dose GnRH agonist regimen (also called 'mini-dose' protocol) and (b) the antagonist protocol.

The 'mini-dose' protocol

This protocol utilizes a micro-dose (50 µg twice daily) of leuprolide acetate and gonadotropin stimulation (150–450 daily IU) per day starting from the second day of the cycle (see Fig. 25.4). Later adjustments such as stepping up the dosage of gonadotropins are the same as in the long protocol. The short protocol is an

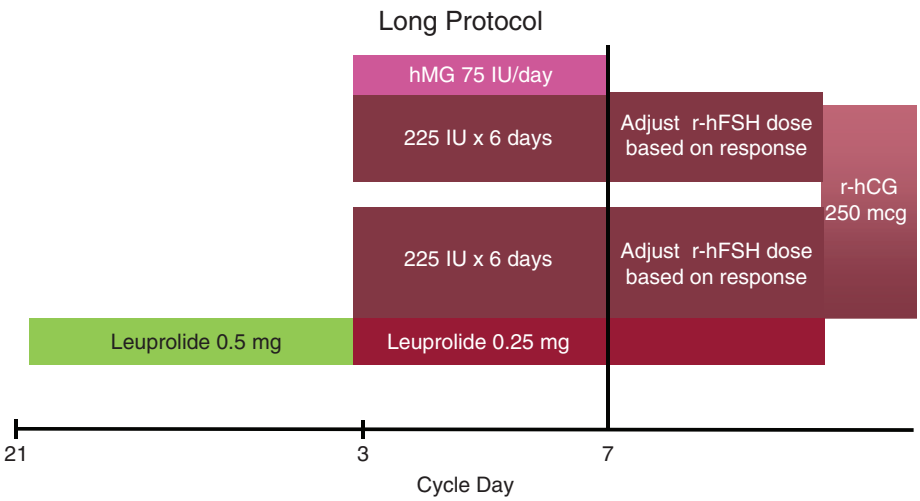

Figure 25.3 A diagram demonstrating the long luteal phase protocol.

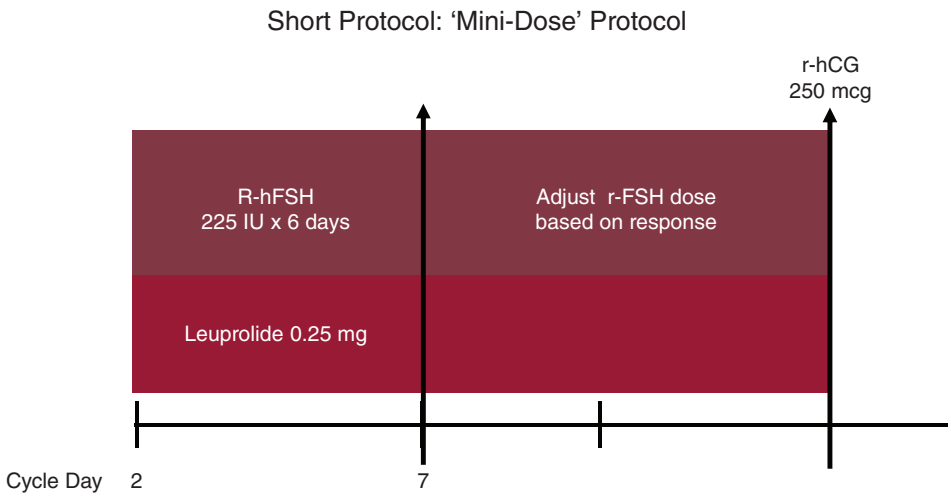

Figure 25.4 A diagram demonstrating the short protocol – 'Mini-Dose' Protocol.

alternative that takes advantage of the initial 'flare-up' phase of the GnRH agonist. A systematic review including 22 trials concluded that the pregnancy rates achieved with the long protocol were slightly higher than that of the short regimen (OR 1.27; CI 1.04–1.56) overall. Because this study did not control for diagnosis and other prognostic factors, these results may not translate to all women [17]. The short protocol generally improves follicular response and lowers cycle cancellation rates in poor responders, although pregnancy and live birthrates remain relatively low in this patient population [18].

Antagonist protocol

Gonadotropin-releasing hormone (GnRH) antagonists were introduced in recent years in ovarian stimulation for ART to exert an immediate inhibition of a premature rise in LH. Because of the immediate effect they can be administered when criteria suggesting a risk for an LH surge are met, i.e. with a leading follicle size of 13 mm, or estradiol level ≥ 400 pg/ml.

The two GnRH antagonists commonly used are ganirelix and cetrorelix. For both, the minimum effective dose to prevent a premature LH surge is 0.25 mg daily subcutaneous administration [19]. The antagonist treatment protocol can be 'fixed' or 'flexible'. In a fixed protocol, the administration of antagonist begins after 5–6 days of gonadotropin stimulation regardless of the follicular size or estradiol level. In a flexible protocol, the initiation of antagonist treatment is tailored to the ovarian response, and the patient would start the antagonist cycle when the criteria (mentioned above) are met. Four randomized controlled trials have compared a fixed (on day 6) versus a flexible (by a follicle diameter of 13–14 mm) protocol of GnRH antagonist administration, and found no significant difference in live birth outcome [20]. Initial studies had shown a lower pregnancy rate in the flexible as compared to the fixed protocol (odds ratio 0.70, 95% CI: 0.47–1.05) [21]. Once GnRH antagonist is initiated, it is administered continuously until the day of hCG. An alternative option is a single 3 mg dose of antagonist which last 4–5 days [22]. Additional daily doses of GnRH antagonists (0.25 mg) can be utilized if the hCG needs to be administered at a later time.

New protocols

Antagonist during the luteal phase

This protocol has specific applications and benefits in certain types of patients such as those with a tendency to quickly develop a dominant follicle at the beginning of a treatment cycle, patients who are poor responders and patients who are seeking fertility preservation treatment and have limited time for stimulation. The antagonist is started during the luteal phase together with low-dose estradiol for 5–7 days until menses occur. On the second day of menses, both the estradiol and the antagonist are discontinued. On that same day, gonadotropin stimulation is initiated after documenting absence of ovarian follicular activity (no ovarian cyst and low estradiol levels). The COH cycle with gonadotropin and GnRH antagonist follows the same criteria outlined previously.

Natural cycle IVF with in vitro maturation

Recently, there has been an increasing interest in combining natural cycle with in vitro maturation (IVM) as a new method of obtaining oocytes for IVF. Since natural cycle IVF alone (i.e. without IVM) has the risk of no oocyte retrieval or cycle cancellation because no embryo is available for transfer, the association of IVM has an added benefit for this protocol. Advantages of natural cycle with IVM compared with controlled ovarian stimulation include the avoidance of ovarian hyperstimulation syndrome, fewer side effects from medications and decrease in costs. In vitro oocyte maturation was initially shown to be a successful treatment for infertile women with polycystic ovary syndrome (PCOS) [23]. However, the combination of natural cycle IVF and IVM has also been offered to patients who are < 35 years old with regular menstrual cycles and with normal antral follicle count (AFC >7 on each ovaries) [24].

A natural IVF with IVM cycle is initiated with a baseline ultrasound on menstrual day 2–3 which is repeated at 2- to 3-day intervals until the leading follicle reaches 12–14 mm in diameter. At this point hCG, which is the only gonadotropin utilized for this protocol, is administered, and oocyte retrieval is performed 36 hours later. On the day of hCG, estradiol supplement is started (4 mg estradiol valerate if the endometrial thickness is ≥ 6 mm, or 6 mg if the endometrial thickness < 6 mm, continued throughout the

luteal phase). The mature oocytes collected at the time of retrieval are inseminated 2 or 3 hours later by intracytoplasmic sperm injection (ICSI). The immature oocytes (i.e. metaphase I and germinal vesicle stage) are transferred into a culture dish containing 1 mL of maturation medium supplemented with 75 mIU/mL of FSH and LH. After 1 day of culture, all cumulus–oocyte complexes are denuded of the cumulus cells and the in vitro matured oocytes are inseminated by ICSI. Fertilization is assessed 17–19 hours later. Embryo transfer is performed on day 3 after oocyte retrieval with embryos that can be at either day 2 or 3 of development, depending upon the maturity of the oocytes. Progesterone supplementation is also started on the day of oocyte retrieval.

Previous published pregnancy rates per embryo transfer in natural cycle IVF alone vary between 0 and 30% [25, 26]. However, in a recent retrospective analysis, Lim *et al.* have claimed that the combination of natural cycle IVF/IVM and the IVM alone can together reach 50% pregnancy rate [24].

Difficult patients

Polycystic Ovarian Syndrome (PCOS)

A correct diagnosis of polycystic ovarian syndrome (PCOS) is imperative to choose a proper ovulation induction protocol. PCOS, corresponding to the World Health Organization (WHO) II group, is one of the most frequent causes of anovulation and related infertility, affecting 4–12% of the female population [27]. Diagnostic criteria in the United States include hyperandrogenism and chronic anovulation in the absence of specific disease of the adrenal, pituitary and ovary [28]. Patients with PCOS usually have a large number of small antral follicles posed to respond to FSH stimulation but, at the same time, they also have a higher risk of ovarian hyperstimulation, multiple pregnancies and the risk of cycle cancellations.

The mainstay for inducing ovulation in PCOS patients is using an oral agent such as clomiphene citrate in combination with or without intrauterine insemination (IUI). However, in cases where PCOS patients are 'resistant' to clomiphene citrate, the use of gonadotropins represents an alternate therapy. There are two main strategies commonly used – the 'Step-Up' and 'Step-Down' regimens. A Step-Up regimen attempts to induce ovulation at a very low daily dose and after 5 days of stimulation, gonadotropin dosage can be adjusted according to estradiol level and ultrasonography findings. The 'low-slow' doses (37.5–75 IU daily) may require small dosage increments and a longer duration [29]. The Step-Down approach starts with higher doses (150–225 IU daily) which are then gradually decreased after the first 3–4 days in order to promote the development of only the most advanced follicle while withdrawing support from the less sensitive smaller follicles in the cohort [30]. Sometimes the ovarian response to gonadotropins during an IUI cycle is excessive, with an estradiol level greater than 1000 or more than three mature follicles. In these instances it is advisable to convert the IUI cycle to an IVF cycle or to cancel so as to avoid high-order multiple pregnancies.

A typical example of an IVF step-down protocol involves the administration of FSH at a starting dose of 225 IU/day for the first 4 days of stimulation. The starting dose is dependent on the body mass index (BMI): 225 IU/day if the BMI is less than 25 or 300 IU/day if the BMI is over 25. The dose is then decreased and adjusted accordingly. Previous studies have conflicting outcomes between the step-up and step-down protocols. In an effort to optimize treatment with the step-down protocol, Van Santbrink and Fauser have adopted a modified approach [31]. In the first cycle, they applied a dose finding low dose step-up protocol in order to determine the FSH threshold for ovarian response. Then, in the second cycle, the step-down protocol was used and the starting daily dose was the effective response dose of the first cycle increased by 37.5 IU. Comparing the first step-up and the second step-down cycle, no significant differences were found in terms of follicular development and pregnancy rates [31, 32].

In addition to the step-down and step-up management, there are two IVF protocols that are popular with PCOS patients. One protocol involves the prolonged ovarian suppression by using birth control pills the month before ovarian stimulation and then overlapping with a GnRH agonist for the last 3 days of the pill. The second protocol is the use of GnRH antagonist, starting a daily dose of gonadotropins concomitantly with a daily dose of GnRH antagonist medication on day 2 of menses. Treatment with gonadotropins and GnRH antagonist are continued daily thereafter, until the day of β-hCG administration. In a randomized clinical trial that compared the long agonist protocol with the GnRH antagonist protocol, Lainas *et al.* did not find any significant difference in ongoing pregnancy rates [33]. However, this study

demonstrated that the GnRH antagonist protocol was associated with a more rapid follicular development, an earlier rise in E_2 levels and significantly higher levels of progesterone when compared with the agonist treatment group. The clinical relevance of the significant difference in the hormonal profile observed during the stimulation cycle between the two treatment groups is unclear. Certainly, both protocols need to be subjected to larger-scale research in order to establish their effect, if any, on pregnancy and live birth outcomes.

PCOS patients are considered to be high responders prone to having an exaggerated follicular recruitment with a massive ovarian enlargement and markedly elevated serum estradiol concentrations (greater than 3000 pg/ml). Under such circumstances, the risk for severe ovarian hyperstimulation syndrome (OHSS) is substantially increased. The management options for OHSS include cycle cancellation, coasting, proceeding with oocyte retrieval but deferring embryo transfer to a future cycle (i.e. freeze all embryos). Of note, approximately 20–30% of coasted cycles are ultimately cancelled, so other preventative strategies have been used in the past in helping reduce the risk of developing severe OHSS [34]. These measures include ovarian suppression with a larger dose of GnRH agonist (20 units versus 10 units subcutaneously daily) or suppression with oral contraceptive (1 daily for 25 days) prior to ovarian stimulation. Furthermore, given the evidence suggesting that hCG may play a pivotal role in the development of OHSS, a lower dose (i.e. 5 000 IU instead of the standard 10 000 IU) may be important for patients who are judged to be high risk for OHSS. Alternatively, GnRH analogues (i.e. leuprolide acetate 0.5 mg–1.0 mg subcutaneous) rather than hCG might be used to stimulate the LH surge for final oocyte maturation. This approach will only be useful in cycles using GnRH antagonists [35].

Other measures such as prophylactic intravenous administration of 25% albumin (25–50 g) at the time of oocyte retrieval or the administration of cabergoline after the retrieval have also been suggested as a means to reduce the risk of developing OHSS [36, 37]. The studies on IV administration of 25% albumin in preventing OHSS have shown conflicting results [38, 39]. However, a recent meta-analysis of five randomized control trials indicated that prophylactic albumin administration significantly reduced the risk of OHSS (odds ratio of 0.28, 95% confidence interval [CI] 0.11, 0.73). It was concluded that albumin infusion may be expected to prevent one case of severe OHSS for every 18 women who were at risk and treated [40]. Another alternate therapy for reducing the risk of OHSS development in IVF cycles is cabergoline, a dopamine agonist, at doses of 0.5 mg/day or 0.25 mg/day for 2–3 weeks) starting on the day of retrieval. A recent meta-analysis of four randomized trials that included 570 women demonstrated that there was a significant reduction in the incidence of OHSS in the cabergoline group (OR 0.41, 95% CI 0.25–0.66) with an absolute risk reduction of 12% (95% CI 6.1–18.2), but had no significant effect on the severity of OHSS [41]. This study suggested that cabergoline treatment may be able to prevent one case of moderate OHSS for every nine women who were at risk and treated [41]. However, the underlying physiological role of dopamine agonists in preventing OHSS is still unclear. Although previous studies have not shown significant differences in miscarriage rates and initial effects on fetal developments between cabergoline and placebo treatment groups [42–44], there are still concerns about its long-term effects.

Poor responders

Stimulating follicle production in poor responder patients is a challenge. A poor responder is generally defined as someone with low levels of anti-mullerian hormone (AMH), elevated cycle day 3 FSH, low antral-follicle count (AFC) and small ovarian volume. For patients with prior ovulation induction cycles, a poor responder is someone who had three or fewer oocytes and estradiol concentrations less than 500 pg/ml in her previous cycles. The incidence of poor responders varies from 9% to 26% because of various definition parameters used in different studies [45]. Management options include using short protocols, either GnRH antagonist or micro-dose protocol together with higher doses of gonadotropin. Higher doses of gonadotropin stimulation may perhaps generate a more vigorous follicular response; however, doses greater than 450 IU daily provide little if any additional benefit. Another alternative method is using a sequential treatment with clomiphene citrate (50–100 mg daily) and exogenous gonadotropins. This regimen has shown an improved response in some poor responders [46].

Breast cancer patients

Breast cancer affects about 200 000 American women each year, of which 9% are women under the age of 45.

It is the most common cancer in women of reproductive age. Early referrals are crucial because it will provide the patient with an option of fertility preservation (i.e. oocyte or embryo cryopreservation) prior to cytotoxic chemotherapy and/or radiation treatment. Protocols for ovarian stimulation in breast cancer patients need to minimize the rise in estradiol concentrations since experimental data have suggested that estrogen can have an indirect mitogenic and growth-promoting effect on breast cancer cells, especially in tumours positive to estrogen receptors [47–50]. Safer stimulation protocols include tamoxifen alone or combined with gonadotropins, or, most commonly, the use of aromatase inhibitors (for example letrozole) to keep estradiol at very low levels.

Combined letrozole, gonadotropin and antagonist protocols have shown significantly lower peak estradiol levels than standard IVF [51]. Letrozole is started orally on the second day of the menstrual cycle at a dose of 5 mg/day until the day of hCG.150 to 300 IU/day of recombinant follicle stimulating hormone (depending on patient's age and ovarian reserve) is initiated on the third day of the cycle and GnRH antagonist is administered when the leading follicle has reached 13 mm in size or with estradiol levels ≥ 300 pg/ml. GnRH analogue can be administered to trigger ovulation instead of hCG because the latter has a longer half-life and thus may prolong high estradiol levels [52]. Letrozole is reinitiated after oocyte retrieval and continued until the estradiol level falls below 50 pg/ml [53, 54]. Initial follow-up data of breast cancer patients who have used this stimulation protocol did not demonstrate an increased risk of recurrence [55].

In addition to safe protocols, another major issue, when discussing fertility preservation options with cancer patients, is the time available to perform ovarian stimulation before the start of chemo- or radiotherapy. Quite often two weeks is all that is allowed and unless the patient is in the early follicular phase at the time of consultation, it is necessary to resort to more creative and novel protocols. Evidence indicates that there are multiple major follicle recruitment waves during a normal menstrual cycle. The idea of ovarian induction at any time during the cycle (so-called random start) with the utilization of GnRH antagonist has been recently reviewed in a case series report [56, 57]. Furthermore, the random-start ovarian stimulation protocol is plausible especially when the endometrial development is irrelevant as is the case in fertility preservation. The protocol includes a baseline pelvic ultrasound and hormonal evaluation on the first day, and this day can be any day throughout the menstrual cycle. Letrozole (2.5 mg/day), recombinant FSH (150–300 IU/day) and GnRH antagonist are given to the patient on the first day of the stimulation at any time during the cycle (random-start). The total duration of stimulation can last between 9 and 12 days. The three patients in the case review series started letrozole and FSH while on cycle days 11 (late follicular phase), 14 and 17 (early luteal phase) of the menstrual cycle, respectively. GnRH antagonist was provided after 5 days of stimulation for the two patients who were in luteal phase and from the outset of stimulation for the patient in the late follicular phase. In all three cases there were follicles that reached preovulatory stage after 9–12 days of stimulation. Oocytes were collected from each case (range 9–17) and each patient had frozen embryos (range 7–10) [56]. It must be mentioned, however, that as of now, it is not known whether the embryos frozen with random start ovarian stimulation will result in pregnancy rates comparable with those originating from conventional stimulation cycles [56].

There are limited and conflicting data regarding late-follicular or luteal-start of ovarian induction and emergency fertility preservation in the literature. Bedoschi *et al.* described emergency ovarian stimulation in two cases with breast cancer and Hodgkin lymphoma where 12 mature oocytes were retrieved in both cases and all mature oocytes were subjected to ICSI with fertilization and cleavage rates of 83.3% and 70%, respectively [58]. The fact remains that there is still a need for early referral so that patients with cancer are given the option of oocyte or embryo freezing [59].

Conclusion

Great progress in ovulation induction has been achieved in the last 25 years, and during these years many protocols have been introduced into practice and refined. However, despite large experience, there is still no consensus for a treatment algorithm for use in daily practice among all the centres. Each centre has slightly different definitions of high/poor responders, and each physician uses the same or different versions of the conventional protocols that are mentioned in this chapter. The process of ovulation induction is still largely considered an 'art of the ART'. Our goal as physicians is to induce ovulation in a safe and efficient manner. That is, achieving mono-ovulation during an intrauterine insemination cycle and inducing safe

multifollicular development during an IVF cycle with the least risk for developing OHSS and multiple pregnancies. Tailoring each protocol to meet the individual patient needs together with strict monitoring and implementation of cancellation criteria are essential to minimize these risks.

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Chapter 26

Intracytoplasmic sperm injection (ICSI)

Caroline Ross

Introduction

IVF is a well-established procedure for the treatment of most types of infertility, including tubal disease, endometriosis, unexplained infertility and some cases of mild male infertility. However, those couples presenting with severe male factor infertility, as demonstrated by low sperm counts/motility, poor morphology or a combination of all parameters, represent the main cause of failed fertilization in IVF.

As a consequence, a number of methods have been developed to try to assist the fertilization process.

The first technique, Partial Zonal Dissection (PZD), involved making an opening in the zona pellucida of the oocyte by means of a mechanical or chemical breach, thereby allowing sperm enhanced access to the perivitelline space [1]. This was followed by Subzonal Insemination (SUZI), whereby between 3 and 20 motile sperm were injected directly into the perivitelline space [2]. While both these techniques resulted in limited success, fertilization rates remained poor and increased rates of polyspermy were reported. Furthermore, they relied on the availability of an adequate number of spermatozoa with good progressive motility, which was not always achievable [3]. The need for more efficient methods of obtaining normal fertilization led to the development of Intracytoplasmic Injection (ICSI), where a single spermatozoon was injected directly into the ooplasm [4]. The first human pregnancy and live birth using this technique was achieved in 1992 [5].

Since then, ICSI has become the worldwide treatment of choice for couples presenting with male infertility.

Patient selection for ICSI

Prior to embarking on a course of IVF, all new patients are required to have an initial semen analysis performed according to the recommendations of the World Health Organization (WHO) Gold manual [6]. On the basis of these results and their referral history, patients are advised whether to proceed with conventional IVF or ICSI. In some cases, where the results are borderline, couples may be advised to consider dividing the oocytes between both ICSI and IVF procedures provided that a sufficient yield of oocytes is obtained on the day of oocyte collection.

Indications for ICSI are summarized in Table 26.1.

Patient indications for ICSI remain controversial and will vary both between units and countries, perhaps as a reflection of differences in local or national guidelines and funding policies. 'Patient pressure' can also be a significant factor in influencing the decision to perform ICSI, as many patients do not want to risk the perceived possibility of failed fertilization in what may be their only attempt at assisted conception.

While guidelines vary, there are some indications that are absolute in their requirement for ICSI, namely, the use of surgically retrieved spermatozoa (epididymal or testicular) and males presenting with total globospermia as diagnosed by the presence of 100% round-headed spermatozoa lacking an acrosome (which occurs in < 0.1% of the population). Other semen parameters, such as concentration, motility and morphology (excluding globospermia) and high titres of antisperm antibodies have not been demonstrated to affect success rates with ICSI [7, 8]. However, it must be remembered that while performing ICSI, the embryologist will always strive to inject the 'best', morphologically normal, sperm available. Therefore, a more accurate assessment of the effects of sperm morphology on ICSI success would be an evaluation of the single sperm injected [9]. De Vos and colleagues found that injection of morphologically abnormal sperm did result in a decrease in fertilization

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

Table 26.1 Indications for ICSI

▪ Oligozoospermia (< 10 x10^6 /ml)
▪ Asthenozoospermia (< 40% progressive motility (A+B) or < 25% 'A' motility)
▪ Teratozoospermia (< 5% normal using Kruger Strict Criteria), globospermia
▪ Poor post-preparation parameters (< 5x10^6 /ml and/or < 65% progressive motility)
▪ Any combination of these parameters
▪ Antisperm antibodies
▪ Fresh/frozen epididymal or testicular sperm
▪ Ejaculatory disorders (retrograde ejaculation, electroejaculation)
▪ Frozen-thawed sperm with poor post-thaw survival
▪ Previous failed or reduced (< 50%) fertilization

(71.7% vs 60.7%), pregnancy (36.7% vs 18.7%) and implantation rates (20.2% vs 9.6%)[10]. This was supported further by Ryu and colleagues, who found that morphologically abnormal sperm had an increased aneuploidy rate (29% vs 1.8–5.5%) when compared with morphologically normal sperm within the same specimen [11].

The use of ICSI for those men presenting with azoospermia provides varying results depending on the source of the sperm. Using meta-analysis, Nicopoullus and colleagues compared testicular versus epididymal sperm and reported similar cleavage, implantation and pregnancy rates following ICSI. However, when these authors compared the results of ICSI cycles in patients with obstructive azoospermia (OA) and non-obstructive azoospermia, there was a statistically significant improvement in both fertilization and clinical pregnancy rates in the OA group. These data suggested that the source of sperm retrieval was not as important as the underlying aetiology of male infertility when performing ICSI for azoospermia [12].

The use of ICSI for non-male-related cases is outlined in Table 26.2.

Child and associates recommended that ICSI should be performed during In Vitro Maturation (IVM) procedures due to the possible hardening effects on the zona pellucida during the oocyte maturation process. This was further supported by the reduced fertilization rates obtained when routine insemination was performed on IVM oocytes [13].

Table 26.2 Non-male indications for ICSI

▪ In vitro maturation (IVM)
▪ Preimplantation genetic diagnosis (PGD)
▪ Female oncology patients

When performing preimplantation genetic diagnosis (PGD) for single gene disorders, ICSI greatly reduces the risk of DNA contamination caused by surplus sperm attached to the zona pellucida. Without the use of ICSI, the laboratory may incorrectly test the excess sperm DNA and not the DNA from the oocyte or embryo and this may lead to misdiagnosis.

Preservation of female fertility prior to the treatment of cancer involves a cycle of IVF followed by cryopreservation of any resultant embryos. Since the chemotherapy or radiotherapy treatment may render the female infertile, ICSI may be utilized in order to maximize the fertilizing potential of any available oocytes. Furthermore, ICSI minimizes the risk of possible failed fertilization due to an underlying, undiagnosed male factor.

Preparation of sperm samples prior to ICSI

The majority of semen samples for ICSI are processed by density-gradient centrifugation using silane-coated silica particle colloid solutions, which results in a concentrated population of morphologically normal spermatozoa for selection [14]. The quality of the initial sample determines whether it is layered on a double (80%, 40%) or a single (40%) discontinuous PureSperm® gradient and centrifuged at 300g for 20 minutes. The resultant sperm pellet is then washed twice by adding 4mls (first wash) and 2mls (second wash) of media (e.g. Cook Fertilisation Media, Cook, Australia) and centrifuged at 500g for 5 minutes. The final pellet is re-suspended in 50µl to 1ml of fertilization media to achieve a final sperm concentration of $2 - 5 \times 10^6$ /ml. In cases of extreme oligoasthenozoospermia (particularly in some frozen, thawed testicular samples), processing via gradient centrifugation may not be advisable as it may result in the loss of vital spermatozoa from the sample and insufficient sperm for use. In these cases, simple washing via two centrifugations at 500g for 5 minutes are performed and the final pellet re-suspended in 50µl of media, thereby

minimizing the loss of spermatozoa for injection. All samples are then incubated at 37°C in a gassed incubator at 6% CO_2 until required.

Preparation of oocytes prior to ICSI

Following transvaginal ultrasound guided oocyte retrieval, oocytes are cultured for a mimium of 4 hours prior to injection. At least 1 hour after retrieval, the oocytes must be denuded of their surrounding cumulus and coronal cells. This removal is essential to allow both assessment of the maturity and the visualization and handling of the oocytes during micromanipulation. The cumulus and coronal cells are removed by a combination of enzymatic and mechanical procedures. Initially, the oocytes are placed in HEPES-buffered medium containing 80 U/ml of ICSI Cumulase® (Origio Ref 16125000A) or Hyaluronidase (Cook Medical K-SIHY-1-5) under oil; this aids digestion of the hyaluronic acid holding the cumulus cells together. After 30–45 seconds, the oocyte complexes are pipetted in and out of a fine bore glass pipette in order to loosen the weakened cumulus mass and release the oocytes with attached coronal cells. The oocytes are then transferred into media overlaid with culture oil (e.g. Cleavage Media, Cook, Australia) and by repeated pipetting of the oocyte-cumulus mass through fine bore pipettes (only slightly larger than the diameter of the oocyte), the remaining coronal radiata cells are removed. The oocytes are then washed serially through additional drops of media under oil. The exposure time and enzyme concentration must be kept to a minimum, as both of these factors have been linked with parthogenetic activation of the oocyte [15]. The denuded oocytes are then examined under the inverted microscope (×400 magnification) in order to assess their maturation stage. Three distinct maturation stages are found. The majority of the oocytes (85%) are usually in metaphase-II (MII) as demonstrated by the presence of the first polar body, 5% are in metaphase-I (MI) having undergone breakdown of the germinal vesicle but not the extrusion of a polar body, whilst the remaining 10% are in prophase as demonstrated by the presence of a germinal vesicle (GV). ICSI can be performed only on those oocytes that have reached MII and are therefore haploid and able to be fertilized normally. MI oocytes may be further cultured in vitro and will frequently achieve MII after a few hours. GV oocytes require overnight culture in order to reach the MII stage and would only be used for injection in very rare circumstances, where there are no MII oocytes available on the day of oocyte collection. However, results to date have been very poor with only a few reported pregnancies and live births [16].

Equipment required to perform ICSI

The equipment required for performing ICSI is fairly well established in most Assisted Reproductive Technology (ART) laboratories and is built around a specifically adapted inverted phase contrast microscope. The microscope should be fitted with ×4, ×10, ×20 and ×40 objectives to allow both setting up and proper evaluation of the oocytes throughout the procedure. Hoffman modulation contrast is also used to enhance the image, especially when working with plastic dishes.

It is essential that the microscope is equipped with a heated stage to allow all manipulations to be performed at 37°C, thereby minimizing the possible effects of cooling of the oocytes while undertaking the manipulations. Current results suggest that even a small decline in temperature can lead to a time- and temperature-dependent depolymerization of the meiotic spindle. Furthermore, recovery after re-warming is dependent upon the initial degree of temperature decrease and is limited to a proportion of oocytes which subsequently exhibit poor fertilization rates and increased rates of aneuploidy during subsequent development [17].

The ICSI rig has two complete sets of micromanipulators mounted on either side of the microscope with coarse and fine control of the injection pipette on the right-hand side and holding pipette manipulators on the left (Fig. 26.1). The coarse movements are controlled by an electrical manipulator, whereas the fine movements are via a hydraulic remote-control manipulator with hanging joysticks. All the manipulators allow for the three-dimensional movement of the pipettes. Whenever possible, it is recommended that the ICSI rig is mounted on a vibration-proof table in order to minimize any interference from vibrations during manipulation.

The two micro-injectors connected to the pipettes are responsible for holding/releasing the oocyte (on the left control) and aspirating and injecting a sperm (on the right control) (Fig. 26.2). They both consist of airtight glass syringes connected via flexible tubing to the micropipette holders. The tubing can be filled with either air or mineral oil and manipulation is controlled by a micrometer. If an air-filled system is used for

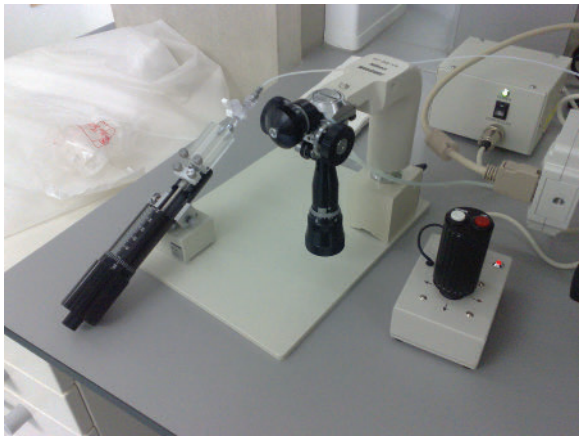

Figure 26.1 Controls on the left-hand side of the system. From left, injector control syringe, fine control unit for holding pipette and coarse control for holding pipette.

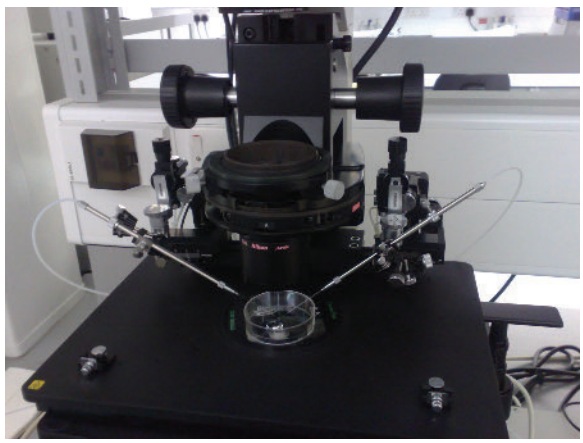

Figure 26.2 Microscope stage showing holding (left) and injection (right) micropipettes.

injecting the sperm, then a screw-actuated syringe (SAS, Research Instruments) may be attached via tubing to the injection pipette. Overall, while the use of mineral oil does offer more precise and smoother control, its use can be messy and is prone to problems, especially when small air bubbles enter the system, become compressed and result in a loss of precision control and result in unpredictable movements within the pipette. As a consequence, many ART units now prefer to use air-filled lines. The pipettes used for micro-injection used to be made by hand on a microforge; this was very time consuming and required a high degree of expertise and patience. Fortunately, pipettes are now made commercially by several companies and can be made to one's own specific requirement if necessary. In Oxford, we use Cook pipettes with a distal tip angle of 30°. The holding pipette (Cook K-HPIP-1030) has an outer diameter of 75µm and an inner diameter of 15µm with a heat polished tip. The injection pipette (Cook K-MPIP-1030) has an outer and inner diameter of 7µm and 5µm respectively and has a sharp spike tip. The sharpness of the spike is very important to ensure a clean and easy penetration of the oolemma during micro-injection.

Setting up and performing ICSI

ICSI rig

The key to good practice while performing ICSI is to ensure that the ICSI rig is set up correctly with care and precision before commencing manipulations. Problems will be encountered during the ICSI procedure if the initial setup of the rig is incorrect. This may result in the oocytes being out of the incubator for longer than necessary and may also result in a higher incidence of atresia following injection.

When starting to set up the ICSI rig it is important to remember to set all graduated scales on the microscope stage motorized drive unit to zero and at an equal height to facilitate proper alignment of the two pipettes. The holding injector screw should also be set to the midpoint. These centring procedures ensure that a full range of movements in all directions are possible during micro-injection.

The holding pipette should always be fitted and positioned first and can then be used as a guide to aligning the injection pipette. There are three main aims when fitting and aligning the pipettes: (i) to ensure a secure mounting in the holders (otherwise there will be a loss of precision control); (ii) the correct alignment in the horizontal plane, both individually and with each other in order to ensure proper visualization and use of the tools during manipulation; and (iii) the equilibration of the pipettes with media before use so that the gametes being manipulated will never come into contact with air or oil and ensuring that the sperm will not become stuck inside the pipette. This is facilitated by neutralizing the pressure in the pipettes.

Once the ICSI rig is correctly set up, it is good practice to leave the micro-injection pipettes in a droplet of media under oil, both before and during the injections, in order to prevent them drying out or becoming blocked.

Preparation of dishes

Preparation of the dishes used for ICSI (such as Falcon 1006) should be performed in a cool area of the Class II microbiological safety cabinet in order to prevent evaporation of the contents of the drops during the process. The dishes should then be equilibrated in a gassed incubator for at least 30 minutes prior to commencing manipulations. The number of dishes required is dependent on both the number of oocytes to be injected and the quality of the available sperm post preparation.

A standard setup consists of a centrally placed 5µl drop of PVP (e.g. Cook K-SIPV-200–5) surrounded by three 10µl 'egg' drops of cleavage media, all overlaid with equilibrated culture oil. To facilitate the setting up and resting of the pipettes between manipulations, an additional dish is prepared containing a single 20µl of culture media overlaid with oil (Fig. 26.3). Just prior to the commencement of the ICSI procedure, 1–2µl of the prepared sperm suspension is added to the PVP drop and the denuded oocytes are placed in the 'egg' drops.

The viscous nature of the PVP solution decreases the motility of the sperm and thereby facilitates their capture and manipulation. PVP also aids control of the injection pipette and helps to prevent sperm becoming stuck in the pipette. Sperm manipulations can be performed without the use of PVP but it does make the procedure technically more challenging, particularly in cases where the sperm demonstrate fast motility.

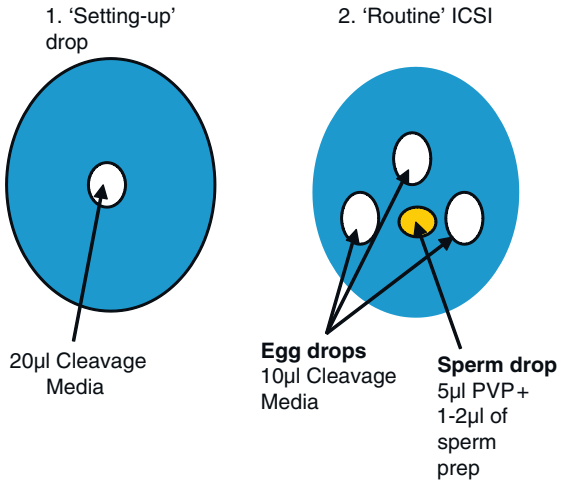

Figure 26.3 Diagram showing dish setup for ICSI: (1) 'Setting-up' drop used for equilibration of the pipettes. (2) Dish setup for performing 'routine' ICSI containing 3 egg drops (Cleavage Media (CM)) and one central PVP drop.

There have been some concerns expressed over the safety of the synthetic polymer PVP and its possible toxic effects on gametes and developing embryos. Dozortsev and colleagues [18] reported that the PVP may interfere with sperm nucleus decondensation. Another cause for concern is that during microinjection, some PVP is also injected into the oocyte along with the sperm. Since PVP is unable to diffuse out of the oocyte and is not digestible by lysosomal enzymes, it remains in the oocyte for a prolonged period of time [19]. Physiological alternatives offering the same properties as PVP have now been developed. These products contain hyaluronate and human serum albumin, both of which occur naturally in the mammalian reproductive tract. Hyaluronic acid (HA) is the major component of the matrix in between the cumulus cells of the mature human oocyte and is involved in the mechanism of sperm selection via specific receptors on the sperm head. It has been demonstrated that spermatozoa with HA-binding characteristics exhibit good nuclear morphology and a decreased incidence of both chromosome aneuploidies and DNA fragmentation. Furthermore, the injection of these sperm can lead to improvements in both embryo quality and development [20]. At present there are three main ready-to-use products utilizing HA technology currently available: (i) a plastic culture dish with microdots of HA hydrogel attached to the bottom of the dish (PICSI®, Origio); (ii) a viscous medium containing HA (SpermSlow, Origio); and (iii) SpermCatch (Nidacon).

A modified setup procedure is used when the quality of the sperm is so poor that either very few sperm are present or a high proportion in postpreparation sample are non-motile. This is commonly seen in cases where the male has very severe oligoathenoteratospermia (OATS) or in frozen/thawed testicular samples. For these cases, the standard setup dishes are augmented with three to four 'long drops' each consisting of 2 × 5µl drops of cleavage media merged together to form one elongated drop into which 5µl of the sperm suspension is added. The dishes are replaced in the incubator and allowed to 'settle' for approximately 30 minutes. During this time, any motile sperm swim out to the edge of the drops and any cellular debris or non-motile sperm sediment to the bottom of the dish. This allows the operator to systematically search through the drops to try and locate any sperm that are either motile or 'twitching'. Once located, these sperm are moved to the PVP drop. As the dish may be outside

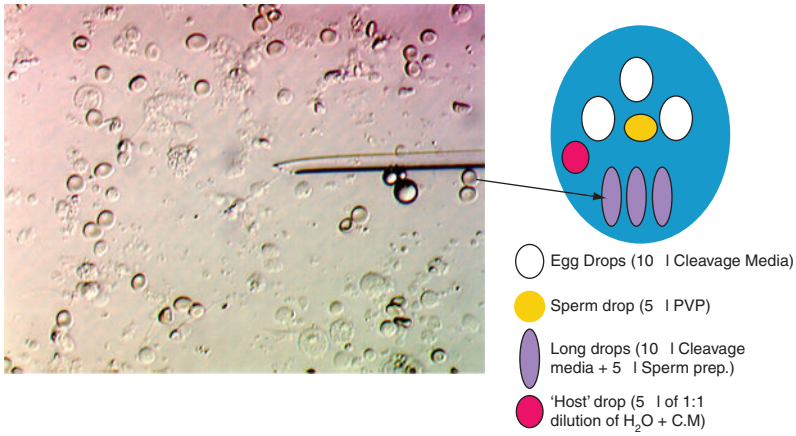

Figure 26.4 Dish setup when using testicular sperm with the addition of three 'long drops' of Cleavage Media into which 5µl of sperm preparation is added and a 'Host' drop consisting of Cleavage Media diluted 1:1 with sterile water.

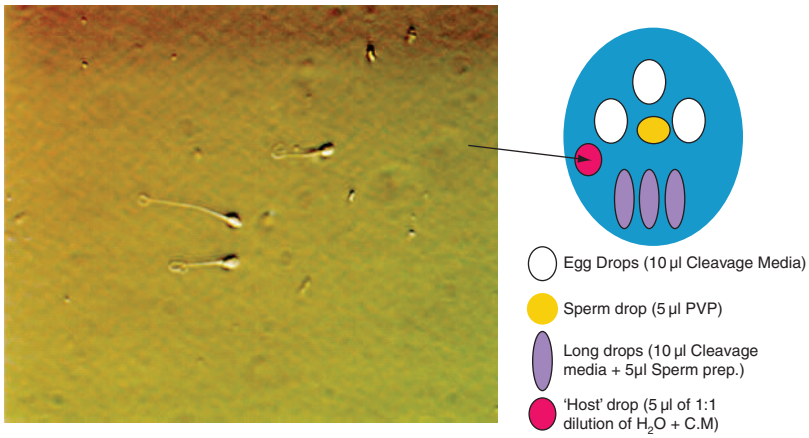

Figure 26.5 Dish setup demonstrating the use of a 'Host' drop to establish viability of the sperm for injection. Host positive sperm are identified by either coiled tails or a swelling at the tip of the tail.

the incubator for a significant period of time while the sperm are being located, it is recommended that the dish is returned to the incubator for >15 minutes to allow re-equilibration prior to placing the oocytes in the culture media drops for injection (Fig. 26.4).

A further adaptation of these dishes is used in extreme cases of male infertility where no 'live' sperm can be observed in the final preparation. The addition of a 'host' drop containing a hypotonic solution of cleavage media and sterile water (1:1 dilution) is used to assess the viability of any immotile sperm present in the 'long drop'. The test relies on the osmoregulatory capability of live sperm and their ability to withstand moderate hypo-osmotic stress. Those live but immotile sperm that are transferred to the 'host' droplet can be identified by a swelling of the tip of the tail or the whole tail curling up. In contrast, dead sperm do not react to this challenge as their plasma membrane is not intact; therefore they do not osmoregulate and their tail remains straight. These 'host positive' sperm should then be placed in the PVP drop and the dish returned to the incubator for ≥ 30 minutes. This facilitates the re-equilibration of the culture media prior to injection and allows time for the osmoregulation of the tail back to normal (Fig. 26.5).

Selection and immobilization of the sperm

Figure 26.6 illustrates the whole ICSI procedure.

Selection of the sperm for injection is usually performed at a magnification of ×400. The primary factors influencing the choice of sperm for injection are their morphological appearance coupled with their swimming characteristics. Preference is usually given to those normal sperm swimming at the periphery of the droplet. However, concerns over the simplicity and subjectivity of this method have led to recent advances in technology which allow the selection of HA bound

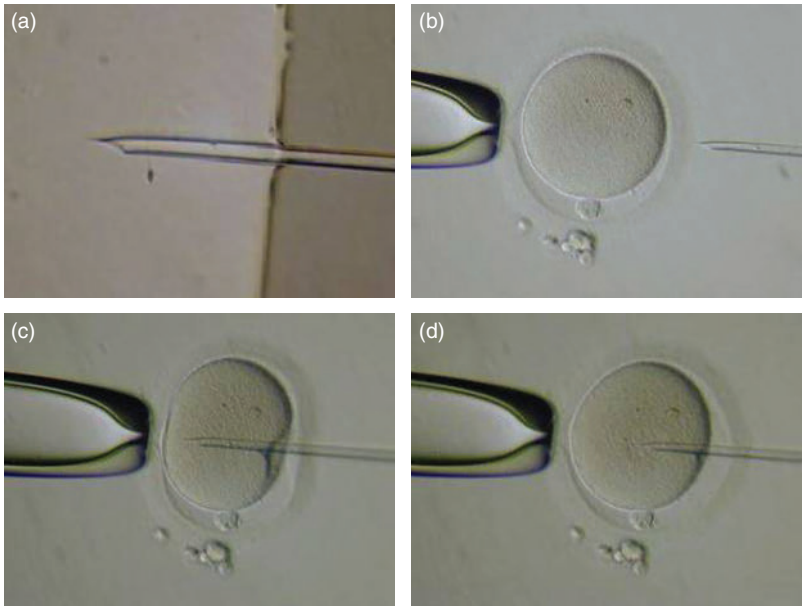

Figure 26.6 ICSI procedure. (a) The sperm is immobilized by breaking the tail by trapping it between the injection pipette and the bottom of the dish and is then taken tail first into the pipette. (b) The oocyte is fixed by the holding pipette with the polar body at 6 o'clock and the injection pipette is focused in the same focal plane as the oolemma. (c) The injection pipette is introduced at the 3 o'clock position, invagination of the oolemma is visible as the injection pipette advances to the centre of the oocyte. (d) The sperm is injected into the centre of the oocyte and the needle is gently withdrawn.

sperm through the use of products specifically designed for selecting the most morphologically normal sperm and naturally selecting sperm for their binding capacities and DNA/chromosomal 'health'. These products were discussed earlier. Another method employed by a small number of ART units is the selection of sperm using motile sperm organellar morphology examination (MSOME) via high magnification (×6600) [21]. Bartov and colleagues reported that out of six sperm subcellular organelles examined (namely the neck, tail, mitochondria, acrosome, post-acrosomal lamina and nucleus), the normal morphology of the sperm nucleus with respect to shape and chromatin content was significantly and positively associated with both fertilization and pregnancy. This led to the application of these MSOME criteria being utilized in the selection of sperm for ICSI, a technique known as intracytoplasmic morphologically selected sperm injection (IMSI). The patients that were selected to undergo IMSI had previously undergone at least two failed cycles of ICSI. This cohort of patients showed significantly improved pregnancy and miscarriage rates [22] and further independent studies have confirmed these observations [23, 24]. While this technique did result in an improved pregnancy rate, it must be stressed that there are limiting factors preventing its implementation in routine clinical practice. The procedure is both extremely time consuming and requires additional specialized equipment to achieve the necessary magnification. Furthermore, it can only be performed by highly trained and experienced embryologists ideally working in tandem.

Once a morphologically normal sperm has been selected using any of the techniques described above, it must then be immobilized prior to injection. This is achieved by aligning the injection pipette perpendicularly above the sperm. The injection pipette is then lowered gently in order to trap the sperm between the pipette and the bottom of the dish. Using a swift movement of the injection pipette across the tail, below its midpiece, results in the required permanent kinking of the tail. Several attempts may be needed to achieve this essential process. An alternative technique is the aggressive immobilization of the sperm by rolling it over the bottom of the dish in a location proximal to the midpiece which results in a permanent crimp in the tail section. The more aggressive method may be of particular use with epididymal or testicular sperm in order to promote membrane permeabilization due to the structural differences in these immature sperm compared with mature ejaculated sperm [25]. Sperm immobilization is believed to be an essential prerequisite for oocyte activation by allowing the release of sperm cytosolic factors via the ruptured membrane and resulting in an increased fertilization rate [26, 27].

Following immobilization, the sperm is aspirated again (tail first) into the injection pipette, in an

attempt to minimize the amount of fluid that will accompany the sperm when subsequently injected into the oocyte.

Orientation and penetration of the oocyte

The oocyte must be held firmly in place with the holding pipette using the minimum amount of suction in order to avoid distortion of the oolemma. The inferior pole of the oocyte should be positioned such that it touches the bottom of the dish, thereby preventing rotation of the oocyte during the injection procedure. In order to minimize disruption of the meiotic spindle, injections are performed routinely with the polar body orientated in the 6 or 12 o'clock position and the injection pipette entering in the 3 o'clock position [28]. Studies using the non-invasive method of polarized light microscopy (Polscope-LC) have demonstrated that the meiotic spindle is not always located under the polar body and in 6.9% of oocytes can even be located in the opposite hemisphere [17, 29]. Furthermore, it has been suggested that in 17% of oocytes the meiotic spindle is found in a position 30° on either side of the injection site and therefore may be more at risk of disruption [30]. Such disturbances of the meiotic spindle may predispose oocytes to perturbation of chromosomal segregation and subsequent aneuploidy leading to maturation arrest, an increased incidence of cell death and lower fertilization rates [31]. The timing of the micro-injection is important to maximize outcome as the appearance of the meiotic spindle is reported to peak at approximately 39–40 hours post hCG [17].

Once the oocyte is in position, the tip of the injection pipette is lowered until it is in the same focal plane as the oolemma. This ensures that the injection pipette remains in the equatorial plane of the oocyte throughout the injection procedure. The immobilized sperm is then advanced to the tip of the bevelled opening of the pipette. Breaching of the zona pellucida is achieved by slowly advancing the injection pipette and with gentle pressure proceeding forward through the inner surface of the oolemma. This creates a funnel-shaped indentation that eventually ruptures at its apex at a position approximately in the centre of the oocyte. However, the membrane is not always ruptured due to this simple advancement of the needle and often minimal suction needs to be applied. Three distinct patterns of oolemma breakage have been described [32].The most common is characterized by invagination of the oolemma followed by spontaneous rupture of the membrane at the centre of the oocyte (74%). In 14% of oocytes funnelling of the oolemma still occurs; however, stronger aspiration or multiple penetration of the ooplasm is required in order to breach the membrane due to its extreme elasticity. The least frequent pattern (12%) consists of instant rupture of the oolemma without any aspiration or formation of a funnel and results in a decrease in both survival and fertilization rates. There is also an associated increase in the abnormal fertilization rate (3PN) due to the reduced ability of the oocyte to extrude the second polar body and the formation of a third pronucleus. While normal fertilization rates are similar in the first two groups, the best embryos are generated following rupture by the first method. This indicates that the developmental capacity of the embryos is influenced by the type of membrane rupture [33].

Once the oolemma is ruptured, there is a sudden acceleration and backwards flow of the cytoplasmic organelles and sperm into the pipette. At this point, suction must be ceased immediately in order to prevent excessive aspiration of the ooplasm and damage to the oocyte. The sperm is then slowly released back into the oocyte accompanied by a minimal volume of media. Ejecting the sperm beyond the tip of the pipette ensures its intimate position amongst the organelles. This both optimizes the interaction of the sperm with the ooplasm and helps to prevent ejection of the sperm from the oocyte during withdrawal of the needle. The bevel of the needle influences the trajectory of the sperm as it enters the oocyte. It is suggested that injecting the oocyte with the polar body in the 6 o'clock position results in the sperm being placed closer to the meiotic spindle as the bevel of the needle directs the sperm downwards. This has been linked to an improvement in fertilization rates and outcome in terms of embryo quality and pregnancy rates [34, 35]. However, many practitioners still prefer to orientate the oocyte with its polar body in the 12 o'clock position due to concerns over the possible disruption of the meiotic spindle as a result of the downwards position of the needles' bevelled opening.

Clinical results with ICSI

At Oxford Fertility Unit (OFU), approximately 40% of all IVF patients undergo ICSI compared with conventional IVF (cIVF) (Table 26.3). Overall, normal

fertilization rates are slightly lower with ICSI (67%) than with cIVF (78%). However, all other parameters, including cleavage rates (98.4% vs 98.5%), clinical pregnancy rates/cycle started (36.1% vs 34.2%) and implantation rates/embryo (24% vs 23%), are comparable. The rate of abnormal fertilization (≥3PN) is lower in ICSI (2% vs 8%) and is attributed mainly to retention of the second polar body during the second meiotic division [36]. The oocyte damage rate following ICSI is 3% which is lower than the 5–10% commonly reported [37]. The use of fresh or frozen/thawed spermatozoa does not influence either the fertilization (71% vs 67%) or pregnancy rates (35% vs 37%). However, the use of ejaculated sperm does result in higher fertilization rates than with surgically retrieved sperm (73% vs 65%), although, no difference is seen in clinical pregnancy rates (39% vs 35%).

In our experience, ICSI is unable to be performed in less than 1% of cases/year and has never been due to a failure to find spermatozoa for injection, even if many hours of diligent searching are required. We have adopted the routine practice of freezing all surgically retrieved specimens and performing 'back-up' freezes on all patients with severe OATS prior to the day of oocyte retrieval. Patients are not allowed to proceed to a treatment cycle until viable spermatozoa have been found in a portion of the frozen sample. Therefore, the inability to perform ICSI is only attributable to the absence of mature oocytes post cumulus removal.

OFU have treated seven patients (12 treatment cycles) with globospermia in the last thirteen years. Compared with normal ICSI results, there was a reduction in both fertilization (26% vs 67%) and cleavage (62% vs 98.5%) rates. However, the pregnancy and miscarriage rates were not affected. These results are consistent with the review published by Dam *et al.* (2006) on globospermia. They concluded that although ICSI was indicated, there was a reduction in fertilization rate, suggesting a reduced ability of the sperm to activate the oocyte. Furthermore, abnormalities of the sperm were also reported. These included disruption of the chromatin compaction, an increased number of cells with DNA fragmentation and in some cases, an increased aneuploidy rate. However, they reported no increase in spontaneous abortions or congenital defects and concluded that the pathogenesis seemed to occur during spermiogenesis, specifically in acrosome formation and sperm head elongation [38].

Table 26.3 Oxford Fertility Unit – comparison of results between ICSI and conventional IVF (cIVF)

	ICSI	cIVF
No. patients	40%	60%
Fertilization rates (2PN)	67%	78%
3PN fertilization rates	2%	8%
Damage rates (injection)	3.0%	–
Cleavage rates	98.5%	98.4%
Clinical pregnancy rate/cycle started	34.2%	36.1%
Implantation rate/embryo	23%	24%

Safety of ICSI

Concerns have been raised regarding the safety of ICSI since its introduction in 1992. The primary objections are the invasive nature of the procedure and the fact that it bypasses the natural selection process by the use of sperm that would not normally be able to fertilize an oocyte without ART intervention.

Bonduelle and colleagues [39] identified two types of risk associated with ICSI – procedure dependent and procedure independent. The main risks associated with the procedure itself are related to both the physical and/or biochemical disturbance of the ooplasm or meiotic spindle. These may result in errors in mitosis during early cleavage divisions leading to aneuploidy. Other risks may be linked to the injection of biochemical contaminants or the injection of foreign, sperm-associated exogenous DNA.

The main procedure-independent problems relate to the actual causes of infertility which result in the need to undertake ICSI. Therefore, the focus is now on those subfertile men who not only have an increased frequency of chromosomal abnormalities (either sex-chromosomal or structural anomalies) but also an increased risk of transmission of these defects to their offspring. Aittomäki and colleagues [40] identified that 24% of subfertile males carried genetic abnormalities including chromosome aberrations, Y chromosome deletions and cystic fibrosis transmembrane conductance regulator gene (CFTR) mutations. Klinefelter's syndrome (47, XXY) is the most common genetic abnormality identified in infertile men and accounts for 13% of azoospermic cases [9].

Bonduelle and colleagues reported that 5% of children conceived by ICSI were at an increased risk of inheriting chromosomal anomalies as a consequence of the chromosomal aberrations in their parents, of which the majority were linked to the male partner [39]. Some of these inherited defects may themselves

cause infertility, for example, specific microdeletions in the AZF region of the Y chromosome, which are prevalent in 8.2% of subfertile men, will result in inherited infertility in any male offspring conceived by ICSI [40]. Similarly, cystic fibrosis (CF) is caused by CFTR mutations located on chromosome 7. The rate of CF affecting Caucasians is reported as 1:2500 with a 1:25 carrier rate. While spermatogenesis is unaffected in males who present with CF, 98% are azoospermic due to congenital bilateral absence of the vas deferens (CBAVD) [9]. Using surgical sperm retrieval techniques these men can father children through ICSI. However, offspring will always inherit a mutated allele from the affected male and therefore an increased risk of inheriting CF. The risk of the offspring presenting with CF is dependent upon the carrier status of the spouse, but can be as high as 50% if the male is affected by CF and his partner is an otherwise healthy carrier of a CFTR mutation. Therefore, it is imperative that genetic testing for CFTR mutations is offered to the partners of men with CBAVD so that the couple can make an informed choice prior to embarking upon ART treatment using ICSI [40].

Other procedure-independent risks associated with ICSI are the use of sperm either with a structural defect or with anomalies of sperm-activating factors, the potential for incorporating sperm mitochondrial DNA and age-related anomalies in the female gamete. Not all of these 'risks' are likely to have clinical consequences.

The safety of ICSI compared with cIVF and natural conception has also been evaluated using pre- and postnatal data. Bonduelle and colleagues [39] detected abnormal karyotypes in 3% of ICSI fetuses of which 1.6% were de-novo and 1.4% inherited (1.3% balanced and 0.06% unbalanced). The majority of the inherited anomalies (17/22) were due to a paternal chromosomal structural defect. The frequency of aberrations was significantly increased in the de-novo cohort compared with those in the general newborn population (1.6% vs 0.45%) and was due mostly to an increased incidence of sex-chromosomal aberrations.

Fetal chromosomal anomalies are linked to both sperm concentration and motility. There is a significantly higher incidence of de-novo prenatal chromosome anomalies in the offspring of men whose ejaculates contain sperm concentrations $< 20 \times 10^6$/ml when compared with the offspring of those men whose samples had sperm concentrations $\geq 20 \times 10^6$/ml (2.1% vs 0.24%, respectively). This suggested that paternal sperm concentrations could be utilized as a tool to select couples for prenatal diagnosis. Abnormal sperm motility parameters is a less useful marker for prenatal anomaly screening due to its low detection rate compared with the high percentage of couples that would exceed the threshold for testing [39].

There is some controversy regarding the risk of birth defects after ICSI. Initial reports focusing on the risk of congenital malformations and psychological development of children reported no differences after ICSI when compared with those conceived naturally. However, subsequent studies did identify a higher risk of hypospadias in IVF offspring and attributed this to paternal subfertility thereby implying a genetic aetiology [9]. A recent meta-analysis indicated there was no significant increase in major birth defects in babies conceived through ICSI when compared with cIVF [41].

Nonetheless, there are still concerns relating to specific types of genomic imprinting disorders. Recent reports have been suggestive of a higher rate of Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) in children born after ICSI and that these could be linked with subsequent childhood cancers; however there are sparse data on the frequencies of these events in the literature. Since ICSI is performed during critical times of genomic imprinting, there is the potential for interfering with gene expression. Studies have reported that it is the maternal allele which is affected in BWS and AS and therefore suggests that sperm differentiation is unlikely to be the causal factor. It has been suggested that the disruption of the methylation process in the maternal genome or early embryo is more likely to be as a result of ICSI or some other aspect of the ART technology used, although this is unproven and remains a subject of debate [9, 42].

Conclusion

Since the introduction of ICSI in 1992, the need for donor sperm has been reduced dramatically and now men with even the most severe cases of infertility can father their own genetic child. However, it is important to consider that the oldest child conceived through ICSI is only 18 years old. While initial data on the developmental milestones and rates of malformations are encouraging, we are not yet certain how these children will fare as they progress through adulthood and indeed try to have families of their own. Therefore, it is essential that ongoing, long-term follow-up studies on the

medical/psychological development and fertility of these children (and their subsequent offspring) are undertaken.

There is already evidence to support an increase in de novo autosomal and sex chromosomal aberrations. Therefore, it is imperative that all couples with severe male infertility are offered genetic evaluation both before and after ICSI in order to improve the success rate of ART and also to avoid the birth of children affected with a severe genetic disease. Moreover, there are still many unknown causes of male infertility and more research is required to allow better understanding and definition of the risks of transmitting infertility or other genetic anomalies to future generations.

Therefore, in order to improve the success rate of ART and to avoid the birth of children affected with a genetic disease, it is imperative that all couples with severe male infertility are offered genetic testing and counselling both before and after ICSI. Furthermore, there are still many unknown causes of male infertility and more research is required in order to acquire a better understanding and definition of the risks of transmitting infertility or other genetic anomalies to future generations following ICSI.

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Chapter 27

Embryo culture

Karen Turner

Introduction

The primary objective of an embryologist is to provide a controlled, stress-free environment for gametes and embryos in order to obtain healthy, good-quality embryos with a maximal potential for development through to implantation and ultimately live birth.

Culture of the human embryo is a complex subject. A number of different factors are required to be in harmony in order to achieve the optimal conditions in which to grow human embryos until the point of transfer. These factors may be: environmental, like laboratory air quality, temperature or gas phase; physical, like the types of incubators used or the culture vessel; or chemical, like the type of culture media (Fig. 27.1). This chapter will examine each of these factors and their individual impact on the growth and development of the human embryo.

Environmental factors

Air quality

The European Union Tissues and Cells Directive has now made it a requirement for IVF laboratories to ensure that certain air quality standards are met. Safety cabinets (i.e. critical work areas), in which gametes and embryos are manipulated, must meet an air quality of Grade A (the highest grade). The background laboratory air environment is required to meet an air quality at least equivalent to Grade D (the lowest defined grade). The evidence for such stringent rules on air quality with regard to IVF is lacking, though the principle of a clean air environment for embryos that do not have their own integral purification systems is a good one.

Anecdotal evidence for the impact of air quality on outcome in the IVF laboratory has existed for many years with descriptions of a deterioration in results coinciding with nearby construction or painting work. Hard data on this, however, are much harder to find. The concerns centre on volatile organic compounds (VOCs) that are dissolved in the atmosphere (e.g. from paint, adhesives, plastics, etc.). These are not filtered out by pore filtration, like HEPA (high efficiency particulate air) filters. It has been demonstrated that the air quality in an IVF laboratory can have higher levels of VOCs than outside air and the interior of newly purchased incubators can have particularly high levels [1]. It is therefore important to allow a period of time for the off-gassing of any new equipment/consumables that will come into contact with gametes or embryos before use. The impact of VOCs on embryo culture has been demonstrated by the addition of aldehydes to culture media. This showed that the growth of mouse embryos was significantly affected, with differing concentrations of aldehydes having an impact on cleavage rates, embryo development to the blastocyst stage or killing the embryos outright [2]. Increased nitrogen dioxide has been consistently associated with lower live birthrates [3].

Depending on the type of incubator being used and the gas combination setup, the environment inside an incubator will be made from a combination of that being supplied via the gas cylinders and room air. In incubators employing 5–6% CO_2 in air, the latter can make up some 95% of the gas volume. Given its direct proximity to the gametes and embryos, the quality of this air is therefore important. Gas cylinders are available from industrial-level purity to medical or pharmaceutical grade and should therefore be selected with caution, as this could mean that they are filled in an uncontrolled environment which may contain high levels of noxious substances. Additional precautions like the use of in-line filters which contain activated

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

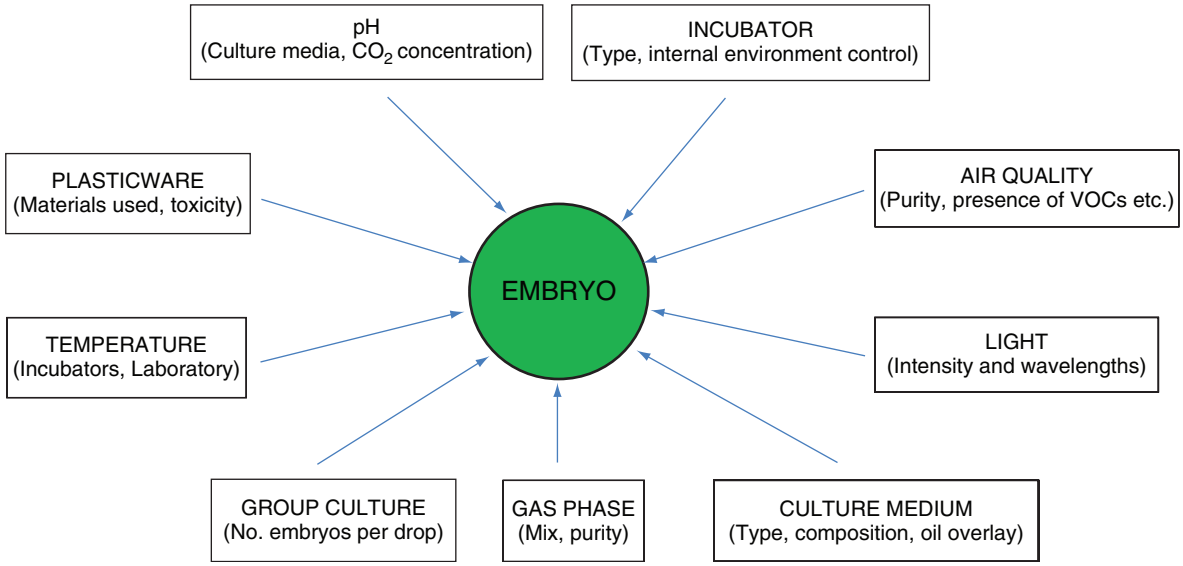

Figure 27.1 Environmental, physical and chemical influences on the human embryo in vitro.

carbon and HEPA filtration to remove VOCs and other impurities or toxicants within the gas can also be taken.

Finally, the use of oil overlay may be an effective way of limiting the impact of any adverse environmental factors. Oil acts as a barrier between the gas environment and the culture media containing the embryo, so if particulate matter is present, it is more likely to get trapped in the oil and so less likely to reach the embryo. Additionally, oil may act to slow gas diffusion, evaporation and temperature loss from culture media during manipulations outside the incubator.

Light

Very little exists in the literature on the impact of visible light on the development of human embryos. In IVF, embryos are exposed to both microscope and ambient light. Visible light has been shown to be an additional stress and to have a deleterious effect on mammalian gamete and embryo development in vitro [4–6] and there is some evidence that human embryo blastulation rates may be improved in low illumination conditions [7]. Blue light (400–500 nm) appears to be much more harmful than longer wavelength light and it has been suggested that the harmful effect of blue light may be the generation of hydrogen peroxide which can cause cellular damage [8]. In view of this, many IVF units consider it prudent to work in low, filtered (i.e. non-UV) light and to additionally minimize the amount of time and observations made on gametes and embryos under the microscope. Light can also degrade the integrity of tissue culture media, so ideally media should be kept in the dark.

Control of pH

Maintaining the pH of the environment of the embryo is an important factor in minimizing stress. Embryo culture media formulations are buffered using bicarbonate and thus, based on the Henderson–Hasselbalch equation, the pH of this media is directly affected by the amount of bicarbonate in solution and the concentration of CO_2 in the atmosphere, as follows:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+.$$

According to this reaction, when CO_2 dissolves in solution it combines with water to form carbonic acid (H_2CO_3), which then reaches equilibrium with the amount of dissolved bicarbonate (HCO_3^-), supplied as sodium bicarbonate, forming hydrogen ions in the process. According to this equation, even small changes to the CO_2 concentration in the incubator will induce significant changes to the medium's pH [9].

Within the incubator, a CO_2 concentration of between 6% and 7% gives an extracellular pH (pHe) of around 7.3. However, pH is dynamic and is also influenced by specific media components such as

Table 27.1 Based on Cook and Sage media

	Fertilization Media Day 0–1	Cleavage Media Day 1–3	Blastocyst Media Day 3–5
Glucose (mM)	Yes (2.8–3.0)	Yes (0.1–0.3)	Yes (2.8–3.0)
Pyruvate (mM)	Yes (0.2–0.3)	Yes (0.2–0.3)	Yes (0.1–0.2)
Non-essential amino acids	Yes	Yes	Yes
Essential amino acids	No	No	Yes
Vitamins	No	No	Cook: No Sage: Yes
pH	7.4 ± 0.1	7.3 ± 0.1	7.4 ± 0.1
Osmolarity (mOSm/L)	Cook: 285–295 Sage: 257–273	Cook: 285–295 Sage: 257–273	Cook: 280–290 Sage: 257–273

lactate, pyruvate and amino acids and their association and disassociation in solution [10]. These, in turn, can be influenced by things like temperature [11] and will vary, along with the amount of bicarbonate present, between the different commercial media available. Thus, to directly assess the pH of culture media within a laboratory's own culture system, a pH meter can be used instead of measuring the CO_2 concentration in the incubator. Use of a pH meter is generally considered a more accurate means of assessing pH. However, it is much harder to measure, particularly in microdrops. If the pH is measured in this way, it is critical to ensure that this is done using appropriate probes and controls. Furthermore, it is essential to have the temperature of both the medium to be measured and the probe at 37°C, and measurements need to be performed quickly to minimize any loss of gas and thus alterations to pH. If, after measurements have been performed, adjustments to the pHe are required, this can be achieved by adjusting the CO_2 concentration in the incubator. For example, a decrease in the pHe is achieved by increasing the CO_2 concentration.

It has been shown that alterations to the culture medium's pH (i.e. the pHe) result in alterations to the intracellular pH (pHi) of the embryo [10]. It is important for embryos to maintain their pHi in order to maintain intracellular homeostasis. This value is generally recognized as being approximately pH 7.1 [12]. Studies have shown that either an increase or a decrease in pHi by as little as 0.1–0.15 pH units can significantly affect embryo metabolism and the developmental competence of the embryo to the blastocyst stage as well as subsequent fetal development [9, 13]. The cumulus cells surrounding the oocyte have been shown to have a pHi regulatory function [14] and thus denuded, mature oocytes have a reduced ability to regulate pHi. Such oocytes are therefore very dependent on the pHe and thus this may be particularly pertinent in procedures such as ICSI and IVM where the protective cumulus cells are either deliberately removed or have varying amounts of unexpanded cumulus [15].

The recommended pHe of commercially available media, which will be based on their ingredients, is shown in Table 27.1 and is in the range of 7.2–7.4. This is set slightly higher than the pHi of the embryo to help counter the acidification that occurs as a result of embryo metabolism. The recognized practice is to fertilize oocytes in a slightly higher pH, culture Day 1–Day 3 embryos in a slightly lower pH and culture Day 4–Day 6 embryos in a slightly higher pH again. The reasoning for this variation is that there is evidence that higher rates of sperm binding occur at higher pHs than at lower pHs, although the effect of this on fertilization is unclear [16]. Cleavage stage embryos anecdotally seem to prefer a slightly lower pH. Once embryos reach the morula / blastocyst stage, they appear to be better able to regulate their pH, possibly due to the formation of tight junctions between cells [10].

As previously stated, embryo culture media formulations are bicarbonate-buffered, which is effective at maintaining the required pH within the incubator in a CO_2 concentration of between 6% and 7%. However, when the embryo is moved outside the incubator for external micromanipulation, the bicarbonate buffer ceases to work as CO_2 concentrations in air are very low and the CO_2 in the medium escapes.

Thus the pH of the medium rises. The length of time that a dish is outside the incubator should therefore be kept to a minimum. However, where this is unavoidable and likely to be for more than a few minutes, another medium employing a buffer that will maintain the pH in air, at the lower levels of CO_2, should be used. The two buffers considered to provide optimal buffering capacities in air for procedures such as ICSI are 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(N-morpholino) propanesulfonic acid (MOPS). The toxicity of these buffers to human embryos has historically been questioned but it remains unclear how real an issue this is [11].

Temperature

The optimal temperature for culture of human embryos is widely believed to be 37°C, reflecting core body temperature. Calibration and control of equipment like incubators, workstations, heated stages and tube heaters, to ensure the temperature is maintained at this level throughout embryo culture is a routine part of any quality assurance programme in the IVF laboratory.

The effect of temperature fluctuations on human oocytes has shown that the meiotic spindle is extremely temperature sensitive and its organization is rapidly disrupted by cooling [17]. Meiotic spindles are crucial for normal chromosome alignment and separation of chromosomes during meiosis, and thus for normal fertilization. Disruption of the spindle will increase the risk of aneuploidy, which is one of the most observed patterns of abnormal fertilization in the human (see Chapter 34). A reduction of the temperature to just 33°C is sufficient to result in the depolymerization of spindles within 10 minutes. At lower temperatures, the spindles depolymerize even more rapidly, and their recovery after rewarming is also related to the degree of cooling and the length of time oocytes spent at the lower temperatures [18]. A recent study suggests that increasing the frequency and prolonging the duration of microscopic observation of developing embryos in vitro is detrimental to blastocyst formation and viability [19]. The effect of overheating should also not be ignored with spindle disruption occurring following increases of more than 38°C [20].

The process of IVF and ICSI necessarily involves the manipulation of oocytes and embryos, especially during ICSI, and thus some temperature fluctuations are inevitable. It has been shown that when culture vessels are placed outside an incubator, there is rapid cooling but only slow rewarming of the culture medium and this can depend on the type of incubator used. Additionally, there are large measurable differences in the ability to maintain the set temperature of culture media that depends on the stage warmer, volume of media and use of vessel lids [21]. It is therefore very important to be mindful of the potential deleterious effect of any such manipulations on embryos and keep these manipulations to a minimum, both in time and frequency. A relatively new approach, allowing frequent embryo observations without excessive exposure to suboptimal environmental conditions, is to employ a combined incubator and microscope system, permitting visualization of oocytes and embryos without removal from the incubator.

Recently, the concept of core body temperature being optimal for embryo development has been challenged with data suggesting that gametes and early embryos function in vivo at a lower temperature than core body temperature [22]. However, the significance of this for in vitro culture of human embryos has yet to be tested.

Low O_2 environment versus high O_2

Physiological conditions in vivo would suggest that the optimal environment for the culture of human embryos is at a temperature of 37°C, a CO_2 concentration of 5–6% and an O_2 concentration of 5%. While there is general agreement in the literature with respect to temperature and CO_2, there is far less agreement on the optimal concentration of O_2. Animal studies show that the concentration of oxygen in the oviduct is around 2–8%, with that in the uterus being even lower, at around 1.5–5% [23]. The evidence for improved embryo development in a reduced O_2 environment in several mammalian species is reasonably strong [24]. However, the case for human embryos has been more controversial. Despite the evidence from animal work, human embryo culture has traditionally used an atmosphere of around 20% oxygen in preference to 5%. This was due to a number of studies being unable to show any statistical differences in pregnancy rates or live birthrates using a low O_2 verses a high O_2 environment [25, 26]. In the absence of any improvement, clinics opted for the simpler and cheaper 20% O_2 incubator environment (i.e. air) as the gas balance for 5% CO_2. However, these studies were generally on embryos where the transfers were performed on Day 2 or 3.

More recently, interest in a low O_2 culture environment has ignited with improvements in culture media and the concomitant move to blastocyst culture. It is claimed that while human embryos will tolerate high O_2 concentrations, this environment promotes the generation of cytotoxic, reactive oxygen radicals that are detrimental to the embryo, particularly in extended culture to the blastocyst stage [27, 28]. Increasingly, the evidence for a low O_2 environment being beneficial for the culture of human embryos to the blastocyst stage is becoming stronger. There is evidence of improved blastocyst development [29], improved numbers of cells present [25] and a marked improvement in birth rate [30].

Physical factors

Incubators

There are two types of incubators available for human IVF embryo culture: front loading, conventional types, e.g. Sanyo, Forma, Heraeus, and top loading, mini incubators, e.g. Cook, Origio (Fig. 27.2a and b). In considering the type of incubator to be used, the overriding aim is to minimize disturbance to the embryos' environment and conditions. Specifically, this is the temperature and pH.

If using the larger, more conventional style incubators, it is preferable to use one designed with individually opening inner doors to minimize fluctuations in gas concentration and temperature. This enables the embryologist to access the patients' embryos that they specifically require. In front loading incubators, the concentration of CO_2 is electronically controlled, utilizing a valve that controls the inflow of CO_2 until the chamber reaches the desired gas concentration. A sensor constantly measures CO_2 in the chamber and initializes an inflow of CO_2 if the concentration falls below the set point. Incubators that employ infra-red sensors, as opposed to thermal conductivity sensors, are more responsive and so regulate the internal chamber of the incubator more effectively.

More recently, top loading, mini incubators have become available. These have much smaller chambers and so employ direct heat transfer from the chamber to the culture vessel. Recovery times are therefore much quicker. Experimental evidence suggests that both the temperature and oxygen tension recovery times after a 5-second door opening/closing procedure were substantially improved in the mini incubator compared with the conventional incubator. In turn, the ability of the type of incubator to maintain a stable microenvironment appeared to significantly influence the formation of good early-stage embryos and the subsequent blastulation rate [31]. However, the converse of this is that heat loss is also much faster, particularly in the event of an equipment problem or power failure.

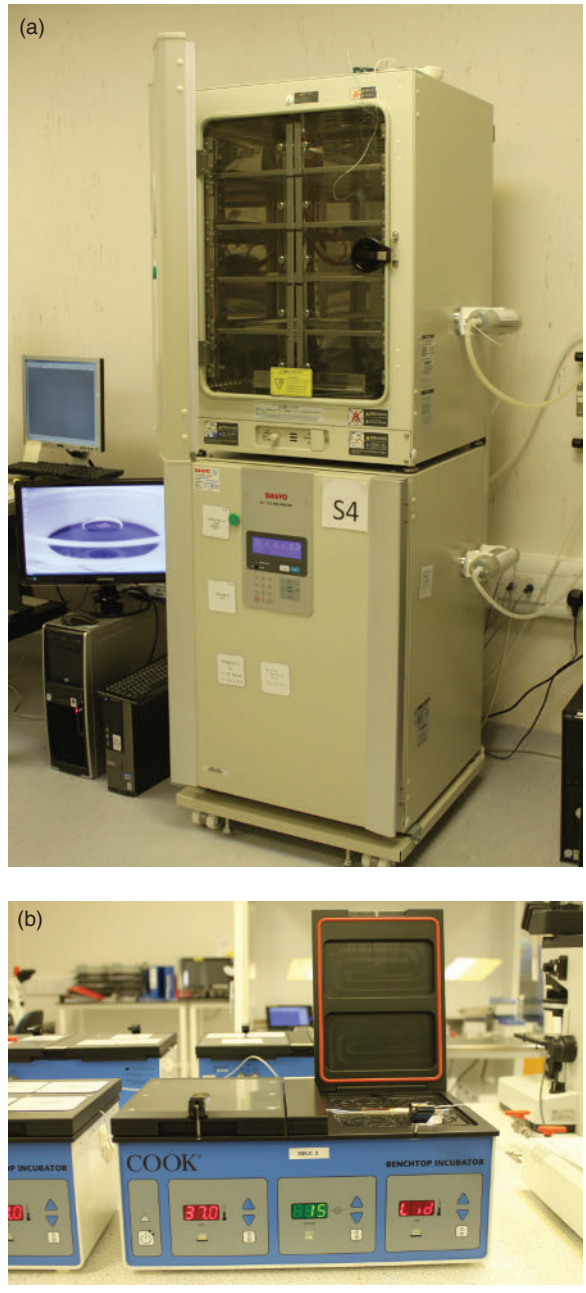

Figure 27.2 (a) Front loading, conventional type of incubator with individual inner doors. (b) Top loading, mini incubator showing small chamber for direct heat transfer.

In deciding number and types of incubator to purchase for human IVF, it is important to consider the number of door openings to try to minimize the amount of times access is required to an incubator that has embryos within. Any incubator can have variations in temperature, depending on the location within it, for example, on different shelves, front or back, left or right. Therefore, this should be mapped prior to use in order to have an understanding of any culture locations that should be avoided. Generally, incubators with an air jacket are considered to be less susceptible to temperature fluctuations than those employing a water jacket. Additionally, different incubators are designed to take different gas supplies. An incubator running at 5–6% CO_2 in air requires a supply of CO_2 gas cylinders only. However, an incubator running at 5–6% CO_2 in a low O_2 environment requires a supply of N_2 as well as CO_2 gas cylinders. This is because a low O_2 concentration is achieved by reducing the 21% O_2 content that is naturally present in air to 5% by an inflow of N_2. Given the smaller volume of internal space, some of the mini incubators employ the direct use of pre-mixed gas (e.g. trimix – 6% CO_2, 5% O_2, balance of nitrogen) which may minimize pH changes.

Consumables

Historically there have always been concerns about the potential variation between different manufacturers of plasticware and different batches. This was due to differences in the materials used during manufacture and, as discussed earlier, the fact that plastic (i.e. polystyrene) test tubes, dishes, flasks and storage vessels all release noticeable levels of styrene and other VOCs that could have a detrimental effect on the viability of human embryos.

Today, companies are generally more aware of the IVF market, so plasticware is available that is designed and sold specifically for this purpose and comes with evidence of more rigorous testing such as CE marking and MEA testing. While these tests, in particular the mouse embryo assay (MEA), are known to have their limitations, they are currently the best available. Therefore, wherever possible, such products should be used.

Reliable in-house testing for toxicity of products can be difficult and expensive. A cheap and easily accessible test that IVF units can use is the human Sperm Survival test [32]. This uses the ratio of the percentage progressive motility of the test sample to the percentage progressive motility of the control sample after 24 and 96 hours. A product that reduces or stops the cell metabolism responsible for motility in human spermatozoa is obviously to be avoided in human IVF. A recent study by Nijs *et al.* [33] demonstrated that 13/36 IVF products tested reprotoxic using the Sperm Survival test with the worst offenders being types of unpowdered gloves, particular types of tubing, condoms, needles or embryo transfer catheters used for clinical procedures and some petri dishes used in the IVF laboratory. The negative impact of reprotoxic products on implantation or ongoing pregnancy rates has been documented [34].

The source of the embryo toxicity is related to the composition of the materials used for manufacture, with the main ones being latex (gloves, oocyte needle tubing), nitrile (gloves), polyisoprene (gloves) or PVC (petri dishes) components. It is well documented that gloves are a major potential source of cytotoxicity and that the toxic substance can be transferred to other products like embryo transfer catheters, even after brief contact [32]. Therefore, it is good practice to test any such items prior to their introduction in the IVF laboratory and to use those that are least toxic and IVF specific.

Chemical factors

Metabolism of the human embryo

The human embryo undergoes a number of physiological and metabolic changes throughout the first 5 or 6 days of its development as it travels down the female reproductive tract prior to implanting into the endometrium. Following fertilization, the pronucleate embryo has a limited ability to utilize nutrients and as a result has a preference for pyruvate as its primary energy source. At this stage it has low metabolic activity and exhibits relatively low levels of oxygen consumption. Although glucose is consumed by the embryo at this stage, it is at relatively low levels [35]. The early embryo also appears to have a preference for non-essential amino acids to be available for it to utilize. Although the embryo is able to develop in the absence of these amino acids, studies have shown that cleavage rates increase in their presence up to the 8-cell stage [36].

Activation of the embryonic genome begins at the 4–8-cell stage in the human and at this time the embryo's metabolic activity begins to increase. The developing embryo is increasing in cell number, protein synthesis increases and as a result, so does the

energy and nutrient requirement. The human embryo increasingly utilizes glucose, in addition to pyruvate, as development proceeds [35]. Post-compaction, at the blastocyst stage, the embryo is utilizing high levels of oxygen and glucose as well as other sources of energy. Non-essential amino acids also continue to be important post-compaction for blastocoel formation and hatching. Additionally, essential amino acids are also required at this time to stimulate cell division and increase development of the inner cell mass in the blastocyst [36]. However, a cautionary note is that these findings on amino acid requirements by the preimplantation embryo may not be relevant in the human [37].

Studies have shown that the available nutrients within different regions of the fallopian tube and uterus reflect the embryo's nutritional requirements. Levels of pyruvate and lactate are relatively high within the fallopian tube while glucose is relatively low. Non-essential amino acids are also present in relatively high concentrations within the tube. Conversely, within the uterus, levels of glucose are much higher while those of pyruvate and lactate are low [38].

Culture media

Historically, single culture media formulations have been employed for embryo culture, irrespective of the stage of development. The basic composition of any media is purified water, salts, nutrients, protein source and a buffer. Recent years have seen the introduction of more complex media formulations which seek to replicate the fine balance of molecular compounds within the female reproductive tract. These 'sequential' media have varying energy substrate concentrations and incorporate amino acids and more complex protein sources that reflect the balance of nutrients in the different regions of the reproductive tract. The different media formulations are used at different stages of gamete and embryo culture and coincide with the changing metabolic requirements of the developing embryo. The use of such media has enabled extended in vitro culture to the blastocyst stage with concomitant improvements in embryo quality and fresh embryo transfer outcome [39].

There are several different types of culture media available commercially. Not all of these media are sequential. Understanding the composition of culture media and the effect that individual constituents can have on embryo development is an important factor in being an embryologist, enabling an informed decision on what medium to culture embryos in and how best to optimize a system. Examples of sequential media are produced by companies like Cook, Origio, SAGE and Vitrolife. However, as Table 27.1 shows, there is no agreement on the exact composition of these media or the concentration that any of the components should be present in. In addition, almost all media require supplementation with chemically undefined or partially defined factors such as albumin or serum. Even the optimal osmolarity for development of human embryos in culture is unclear.

A sequential media suite usually consists of sperm preparation medium, two or three types of gamete/embryo culture media, media for manipulation of gametes and embryos outside of the incubator and cryopreservation/thawing media. Each series of media is developed to work together by sharing a common basic formulation in order to reduce stress on the embryo that could otherwise be induced by moving from one medium type of the series to another. In this respect, it is inadvisable to mix different phases of media from different sequential systems.

Media formulations for culture of embryos are based on CO_2 concentrations of 5–6%. Generally two types of media are used to culture embryos: one type is used to culture embryos from Day 1 to Day 3 of development, and another is used for extended culture from Day 3 to the blastocyst stage on Day 5 or 6. Table 27.1 shows the composition of two of the sequential media available within the UK. Glucose concentration is initially high in media used for fertilization to aid cumulus oocyte complex and sperm cell metabolism. It is relatively low in media used to support cleavage stage development due to the embryo's preference at this time for pyruvate, but is again high in media supporting blastocyst development as the embryo's metabolic activity and ability to utilize glucose increases. Pyruvate concentration is initially relatively high in media supporting oocyte and embryo development up until the time of compaction, but is decreased in blastocyst development media as the embryo starts to use greater quantities of glucose. With regard to amino acids, non-essential amino acids appear to be required during the cleavage stages of embryo development and hence many companies include these in their fertilization and cleavage media with essential amino acids being more pertinent post-compaction and thus added into blastocyst media.

It is important to be aware that chemical changes can occur in culture media when incubated at 37°C. The most significant of these can be the production of ammonium from amino acids present in the culture medium either by their spontaneous breakdown or by their metabolism by embryos. This production of ammonium can have a serious detrimental effect on embryo development [36]. As a result, a more stable form of glutamine (alanyl-glutamine) is usually added to culture media during its manufacture. An additional precaution is to renew culture media on a regular basis. Therefore, this is usually performed every 2–3 days, providing a balance between the removal of negative effects of media depleted of nutrients or altered in composition and the positive effects of any buildup of autocrine factors that may promote embryo development.

Culture media also requires the addition of a protein source because it is thought to maintain the stability of cell membranes and scavenge any toxic compounds that may be present in the culture media or culture vessels. In practical terms, it acts as a surfactant, facilitating the in vitro handling of gametes and embryos. Traditionally, patient serum was used. However, this practice has a number of major drawbacks, not just in terms of its preparation time and risk of infection to both patients and laboratory staff. Serum is undefined, containing a variety of macromolecules such as hormones, vitamins and fatty acids which will vary from patient to patient and time to time in its composition. Furthermore, Large Offspring Syndrome, which is observed in ruminants born as a result of in vitro culture, has been attributed to the presence of serum in the medium, though fortunately studies have not been able to make such a connection in the human [40].

These days, culture media tend to have protein added in the form of human albumin which makes up the bulk of the protein present in serum. Although it is a relatively defined component of serum, it still contains variable amounts of fatty acids and other small molecules which can influence embryo development. Thus, it is crucial that commercial IVF media manufacturers employ adequate quality control measures to ensure that each batch of media is screened to ensure good mouse embryo development prior to clinical use. While recombinant human serum albumin is available, which would allow for greater standardization of media and remove the inherent risk of using blood-derived products, its incorporation into IVF culture media has not taken place, possibly due to the high cost of manufacture.

Embryo group culture and embryo: volume ratio

During IVF, embryos can be cultured either singly or in groups. Support for group culture comes from the fact that the fate of normally fertilized embryos is usually determined on the last evaluation prior to transfer or cryopreservation with relatively little emphasis placed on previous observations. Culturing embryos singly, however, allows the history of each embryo to be taken into account which can be useful in cases of single embryo transfer where a number of blastocysts are of equal quality at the time of transfer. There are now available embryo culture dishes that allow for group culture of embryos while permitting evaluation of their progress individually.

The evidence for the communal culture of preimplantation embryos in several mammalian species is strong but is not conclusive in the human. The group effect can be increased by decreasing the volume of culture medium. The benefit of culturing embryos in groups is thought to be due to the production of specific embryo-derived autocrine or paracrine factors that promote embryo development. Thus, culture of embryos in small volumes enables these factors to reach sufficient concentrations to have an impact, whereas larger volumes will lead to their dilution, rendering them ineffective. Improved development after group culture has been reported in the mouse, sheep and cow, with the latter two species being monovular like humans. In the mouse and cow, increasing the ratio of embryos to the volume of culture medium stimulates the development of the inner cell mass and increases embryo viability [36].

Microdrop volumes of between 20 and 50ul containing between one and four human embryos are generally used in IVF units worldwide. However, in humans, studies on embryos have not yielded consistent results. Group culture of embryos up to Day 2 after insemination seemed to improve development and lead to higher pregnancy rates [41]. However, no significant influence of either communal growth or incubation volume could be found on embryo development from culture of Day 3 embryos to the blastocyst stage [42]. In this study, single culture of embryos in a small volume yielded apparently the highest,

though not significant, rate of blastocyst formation. Finally, in human IVF, the addition of an embryo with good development to a group with retarded development resulted in improvement in the development rates and pregnancy potential of the retarded embryos [43].

Summary

The importance of the embryologist knowing and understanding the requirements and potential stress factors on an embryo in culture cannot be underestimated. There is evidence that the human embryo can actually develop and adapt to a number of in vitro conditions and therefore it is possible to get away with a suboptimal culture environment, at least in terms of embryo development proceeding. However, the testament to a good laboratory and culture environment (clinical factors not withstanding) is its success rates, especially in terms of implantation rates. An embryo that is capable of successfully developing through to the blastocyst stage does not necessarily mean a developmentally competent and viable embryo.

Future improvements in human IVF and embryo culture are likely to be in the continued development of closed culture devices that act as incubators and incorporate microscopes. Such equipment provides a controlled environment with a constant temperature and stable CO_2 and O_2 concentrations. The addition of a microscope and air locks for hands that allow the embryologist to manipulate the embryos without needing to take them out of this environment means that they are not subjected to unnecessary stress that would otherwise be caused by cooling or pH changes [44]. This is supported by studies showing improved blastulation rates and enhanced blastocyst quality when microscopic examination of embryos on Days 2 and 4 is avoided [19].

For the embryologist, the challenge remains to provide a stress-free culture environment that supports good embryo development to enable the optimal stage for transfer of embryos to the uterus to be determined. There must be confidence in the system in order to allow the selection of the strongest, most viable embryos and to perform elective single embryo transfers on those patients who are at greatest risk of a multiple pregnancy in order to reduce the risk to both mother and baby.

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Chapter 28

Embryo biopsy

Tracey Griffiths

Introduction

Since the mid 1980s the micromanipulation of oocytes and embryos has undergone a steady stream of discovery and technological advances. Chronologically, evolution began when micromanipulation techniques were used to assist fertilization in cases of male factor infertility [1]. The methods used included partial zona dissection (PZD), which uses a microneedle to make openings in the zona pellucida [2], subzonal insertion of spermatozoa into the perivitelline space (SUZI, MIST) [3, 4] and chemical digestion of the zona using acid (zona drilling) [5]. However, these techniques were characterized by poor rates of normal fertilization and were largely superceded by the introduction of intracytoplasmic sperm injection (ICSI) from 1992 onwards [6].

Another methodology associated with breach of the zona pellucida is assisted hatching, which involves drilling a hole in the zona of an embryo [7]. All the methods mentioned above concentrated on getting sperm into the oocyte or allowing the blastocyst to hatch, but ultimately they facilitated the development of blastomere biopsy methods for preimplantation genetic diagnosis (PGD) [8].

The stages at which biopsy can be performed include first and/or second polar bodies; removed sequentially on the day of oocyte collection and on day 1 (after fertilization check) [9] or simultaneously after fertilization check, or the removal of one or two blastomeres from a day 3 cleavage stage embryo [8] or trophectoderm cells from a day 5 or 6 blastocyst [10, 11]. In this chapter all of these will be discussed individually, with the pros and cons of each being evaluated.

Breaching the zona pellucida

Naturally, sampling of genetic material from oocytes and embryos relies on creating a hole in the zona pellucida large enough for polar bodies of blastomeres to be extruded, or to allow trophectoderm cells to herniate out. The hole size is critical; if it is too large there is a risk of several blastomeres being dragged out along with the one being biopsied. Additionally, there have been suggestions that large holes may be detrimental to embryonic development. If the hole is too small, stresses on blastomeres undergoing biopsy may be excessive, leading to cell lysis. In the case of blastocyst biopsy, holes that are too small may inhibit hatching, leading to implantation failure or the formation of trophoblastic vesicles resulting in blighted ovum [12]. Also, further mechanical pinching off of a portion of the hatching blastocyst may increase the likelihood of monozygotic twinning [13].

There are several methods that can be used to breach the zona:

- Manual/mechanical zona dissection
- Acid tyrodes
- Laser
- Piezo-mediated devices

Manual/mechanical zona dissection

Microsurgical procedures using glass microneedles were the first to be implemented and various methods have been described, from simple tearing of the zona using glass hooks [14] to three-dimensional PZD [15]. This is the method traditionally favoured by groups performing polar body biopsy.

The approach is simple and efficient but necessitates the correct positioning, release and rotation of the oocyte, a process that requires extensive skill and experience if it is to be performed successfully.

Slits 20–40μm long by 2μm wide have been described which are ideally suited to allow the introduction of a polar body biopsy pipette. However, a

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

small number of groups are using this method for cleavage stage embryo biopsy as it may have the advantage of protecting the embryo until expansion. This is because it is thought that the zonal flaps close after the biopsy pipette is removed, unlike the actual holes created chemically or with a laser which remain open. Holes may allow premature hatching of the embryo and could lead to the problems highlighted earlier [12, 13].

Acid tyrodes

Localized dissolution of the zona pellucida using a controlled, directed stream of acid tyrodes (pH 2.2–2.6) remains the most widely used approach for cleavage stage embryo biopsy [5, 16]. When properly applied, it can be safe, effective and represents a cheaper option when compared with laser technology, as well as having potential speed advantages over mechanical zona dissection. Figure 28.1 is a schematic representation of zona drilling using acid tyrodes.

The dimension of the hole created using acid is difficult to standardize as it depends on the amount of acid used, the resistance of the zona and the skill of the operator. However, the hole should usually be between 15–20μm [18]. Larger holes, once common in PGD practice, are now avoided.

Laser ablation

The first reported systems used for zona drilling were the argon fluoride excimer with an emission wavelength of 193nm [19], the xenon chloride laser emitting 308nm [20], the pulsed nitrogen laser and then

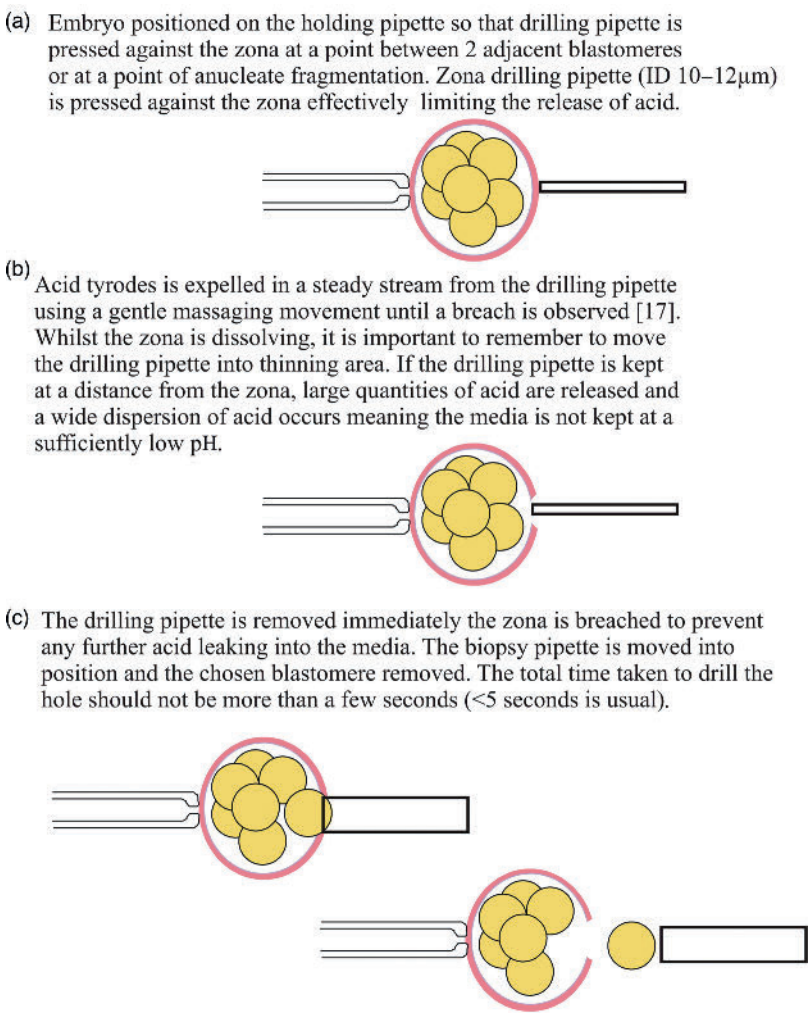

Figure 28.1 Schematic representation of drilling using acid tyrodes.

infra-red systems [21–23]. Problems with the irradiation spectra of some of these systems have meant they are unsuitable for clinical application [24]. Problems include possible damage to the DNA and other cellular macromolecules due to the absorption of UV light or the need for a complex setup that delivers laser energy directly to the target (i.e. contact-mode) to prevent it being absorbed by the surrounding media.

The 1.48μm diode non-contact infra-red laser has emerged as the most acceptable for human use [18, 25–30], as the longer wavelengths of the infra-red range are not absorbed by DNA or media. The size of the hole created can be controlled depending on the type of biopsy to be performed. Drilling should start at the outer edge of the zona and, using short bursts, gradually move inwards keeping the firing position a safe distance from the adjacent blastomeres. It is important to remember that the laser actually creates a trench in the zona which extends above and below the apparent hole. In addition, the use of a laser for zona drilling means that subsequent biopsy can be performed without the need to change pipettes or move to another culture dish/droplet, as is the case when using acid tyrodes.

There are several commercially available laser systems licensed for use in human IVF. These include the Fertilase microlaser (MTM, Montreux, Switzerland), Saturn Active Laser System (Research Instruments, Cornwall) or Zilos-tk non-contact laser (Hamilton Thorne Biosciences), all offering simple, repeatable and controllable zona ablation.

Piezo-mediated devices

These devices harness the piezo-electric effect to transmit a small crystal lattice distortion to the tip of a pipette, driving it forward in a precise and controlled manner. Some work with piezo-mediated drilling has been done in both human and animal models [31]. However, work performed in the mouse has led to concern that excessive use could be detrimental to embryo development.

Published data [18, 28, 30] comparing the use of a non-contact laser (LZD) or acid tyrodes (ATD) for breaching the zona has revealed the following pertinent points.

1. There appear to be more intact blastomeres following LZD and lysis of aspirated blastomeres occurs less frequently. This appears to be related to the impact of the acid during the drilling procedure. The cell abutting the site of zona drilling is likely to suffer some degree of exposure to acid.
2. The overall time required to perform embryo biopsy was in favour of the laser system having less pipettes to set up initially and no swapping between pipettes during the procedure. This has implications for the cost of the procedure and for the impact on the embryo. Ideally, biopsy should be rapid, minimizing the time the embryo spends outside the incubator.
3. Earlier compaction was observed following LZD with a higher percentage of embryos showing compaction by the evening of day 3. Although there were no significant differences between the two methods in the overall number of embryos reaching blastocyst by day 6, blastocyst formation was evident earlier (day 5) in the LZD group.
4. A considerably higher number of ATD embryos were arrested before reaching the blastocyst stage on day 5.
5. There was no difference in cell number between groups of embryos that reached the blastocyst stage on day 5. However, on day 6, cell numbers in the ATD group were significantly reduced. This may be due to the embryo's inability to recover from the temporary but significant effects of the acid [32, 33].
6. The majority of blastocysts had normal spindles but the total number of spindles per blastocyst was higher in the laser group.
7. The majority of morulae that failed to become blastocysts by day 6 exhibited either multinucleated or abnormally shaped spindles [30].
8. Thus far, concerns regarding thermal damage caused by the proximity of the laser beam to the blastomeres appear to be unwarranted, although a truly definitive study, examining biopsy at all possible embryonic stages, is lacking.

After careful evaluation of these findings, the Oxford Fertility Unit (OFU) has opted to perform all stages of embryo and blastocyst biopsy with the use of the Saturn Active laser system which is mounted on a Nikon microscope equipped with Narishige micromanipulators. Figure 28.2 shows the system currently used by OFU. Other laser-based systems for embryo biopsy are available and may function equally well, but have not been fully evaluated in our clinic.

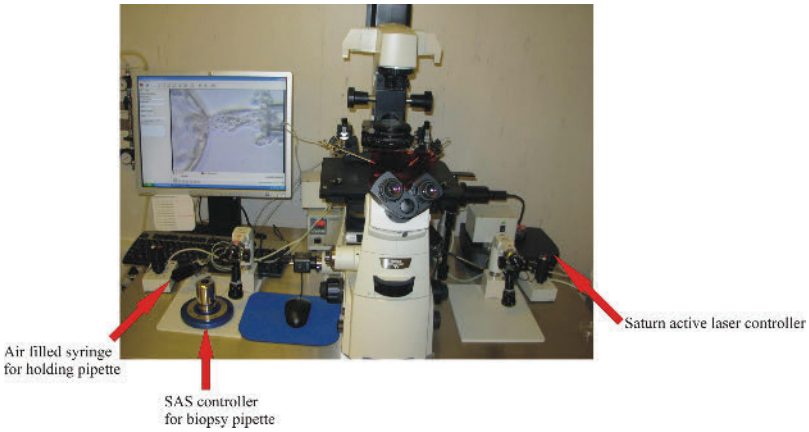

Figure 28.2 Saturn Active Laser System.

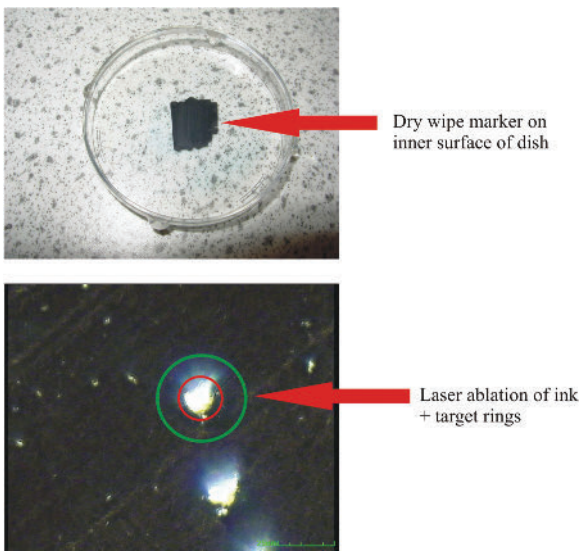

Figure 28.3 Laser calibration dish.

The methodology used in our laboratory will be discussed in detail, however, one of the key elements is the accurate setup and calibration of the laser before use. The Saturn Active laser has its own calibration programme and once this has been performed, a second check is carried out using a biopsy dish which has been marked on the inner surface with a dry wipe marker (see Fig. 28.3). When the laser is fired at the ink it burns a hole showing the ablation area of the pulse and its position inside the target ring.

For PGD cases, ICSI is always performed. For fluorescence *in situ* hybridization (FISH) cases, ICSI is not necessary. For aneuploidy screening using comparative genomic hybridization (CGH) or micro-array chromosomal genomic hybridization (aCGH), ICSI is preferable but not essential (depending on the semen parameters and the stage of biopsy to be performed). However, it is vitally important that the biopsied cell(s) is washed thoroughly. After injection (or following fertilization check for IVF patients) we typically carry out culture using 6% CO_2 and 5% oxygen and COOK sequential media.

Polar body biopsy

A method for the diagnosis of genetic abnormalities of maternal origin [34–36]. The oocyte's first meiotic division results in the extrusion of the first polar body which contains the counterpart of the chromosomes present in the oocyte. Furthermore, analyzing the second polar body will allow verification of the occurrence of crossover between homologous chromosomes. The method has been adapted for the detection of single gene disorders, translocations and X-linked disorders. Because the biopsy uses products formed prior to the fusion of the male and female pronucleus, it is considered as preconception analysis [38] and is therefore legal in some countries, like Switzerland and Germany, where PGD of embryonic blastomeres is currently restricted or forbidden.

It has been shown [37] that although fertilization could be achieved following the use of acid tyrodes on oocytes, there was an inhibitory effect upon embryo development. Since it has been established that both oocytes and polar bodies are sensitive to acid, breach of the zona pellucida in this way is not used for polar body biopsy.

If biopsy is being undertaken for the purpose of PGS, many centres prefer to only take the first polar body. However, in cases of PGD for single gene

disorders it is essential to biopsy and test both polar bodies. The two polar bodies may be biopsied simultaneously or sequentially. In clinics opting for sequential removal of the first and second polar, it is advisable to perform the first biopsy approximately 4 hours after oocyte retrieval, by which time the polar body should be completely detached from the oolemma [38]. Therefore the oocytes need to be stripped using cumulase (ICSI Cumulase Origio Ref: 16125000A) approximately 2 hours prior to the biopsy and returned to the incubator to allow them to recover. It is very important to remove all cumulus cells, as they have their own DNA which could potentially lead to misdiagnosis. Individual ICSI injection dishes (Falcon 1006) are then prepared for each mature oocyte using cleavage media.

The OFU has found that when using the laser for zona drilling performing ICSI first and returning the oocytes to the incubator for approximately 30 minutes before removal of the first polar body maintained high fertilization/cleavage rates and minimal oocyte degeneration. This is despite the concerns that have been raised that performing the procedures in this order may lead to a weakening in the integrity of the oolemma due to the stress of the injection procedure, which may in turn cause lysis when the polar body is subsequently biopsied.

If mechanical dissection is to be the method of choice for zona drilling, the polar body would be removed first and then the oocyte injected through the zonal breach already created. This technique is easily performed by a skilled person, but the learning curve is steep compared to the use of a laser [15].

The second polar body is removed the following morning immediately after the fertilization check through the hole in the zona pellucida already present. Ideally this should be performed 16–18 hours after ICSI; pronuclei will still be visible but the connections between the oolemma and the polar body should have lessened. Great care is needed as these connections may still exist and excessive force during removal may lead to the oocyte lysing.

As an alternative to the three manipulations required in the strategy outlined above, both polar bodies may be removed simultaneously. This reduces the possible stress the oocyte is exposed to during the ICSI procedure because in cases with normal semen analysis, conventional IVF can be performed. As stated previously, all cumulus cells and sperm must be removed when the fertilization check is performed to reduce the risk of misdiagnosis due to DNA contamination. It is also important to note that simultaneous biopsy can only be performed during a time window of approximately 6–>14 hours post-fertilization. This equates to the biopsy being performed 15–18 hours post-insemination/injection. After this time period, the first polar body starts to degenerate, which may again lead to diagnostic errors [34].

Figure 28.4 shows removal of the first polar body using the Saturn Active Laser. We have found this method to be safe, effective, very reliable and reproducible, with the biopsy taking 1–1.5 minutes. It is very important that, if the polar bodies are fragmented, all the pieces are removed and sent for analysis, otherwise an abnormal result may be obtained.

One of the key elements to the success of polar body biopsy is to ensure the polar body and biopsy pipette are aligned in the same plane of focus. The oocyte is held on a holding pipette with the polar

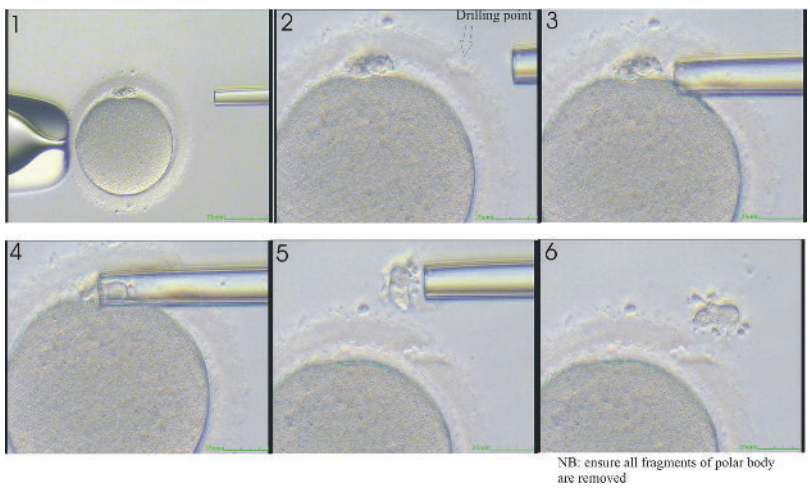

Figure 28.4 Laser assisted removal of 1st polar body.

body positioned at 12 o'clock. This allows the zona to be drilled at the 1 o'clock position, minimizing any potential heating effects on the polar body or oolema. The hole is usually 18–25μm in diameter. We perform biopsy using a Humagen polar body biopsy pipette (Ref: MPB-FP-35). These are 13–15μm, flame polished and blunt-ended, which greatly reduces the risk of damaging the polar body or oocyte. The pipette is gently pushed into the perivitelline space and the polar bodies carefully aspirated into the pipette. If the cytoplasmic bridge is still present between the polar body and the oolemma, it can be weakened by pushing the polar body to the left slightly with the biopsy pipette (assuming the holding pipette is on the left and the biopsy pipette is on the right).

Montag and colleagues 2006 [39] showed that the presence of spindle fibres between oocytes and newly formed polar bodies is a natural occurrence during the meiotic cell cycle. Consequently, if the cytoplasmic bridge isn't easily broken, then the biopsy should not be performed as it could lead to the formation of small ooplasmic droplets which could contain genetic material or the lysis of the oocyte as previously suggested. Some clinics also use polarization microscopy to identify the location of any spindle remnants prior to biopsy.

Due to the limiting factor that analysis of the polar bodies only allows an indirect diagnosis of the chromosomal constitution of the oocyte and provides no insight into the paternal genetic contribution, we find that the majority of couples opt for cleavage or blastocyst stage biopsy instead. In fact we now only routinely consider polar body biopsy in couples with four or fewer oocytes, unless the couple have specifically requested the procedure due to moral or ethical concerns.

For couples with greater than five oocytes we discuss with them the options for either day-3 biopsy or trophectoderm biopsy. Once the day-3 embryo grading has been performed, a final decision is made. If more than eight embryos are good quality 6 –> 8 cells, or they have already compacted, they are cultured on to blastocyst stage, otherwise we proceed with blastomere biopsy.

Cleavage stage biopsy

Currently still the most widely used approach for genetic testing of embryos [16]. One or two blastomeres are removed approximately 64 hours post-insemination or injection. In our unit, biopsy is only performed for routine PGS cases if the embryo has at least six cells. However, for PGD cases, biopsy would be performed on an embryo with four or more cells. The reason for the slight difference in strategy is that PGS is only valuable if testing can be performed without compromising embryo viability (embryos are likely to need at least 5–6 cells if they are to be biopsied without impacting their implantation potential). In the case of PGD, the primary reason for testing is avoidance of an affected pregnancy, rather than maximization of pregnancy rate, so it is worth testing as many embryos as possible even if viability is compromised. Testing the greatest number of embryos possible provides the best chance that at least one healthy embryo will be identified.

Cook biopsy media (calcium/magnesium free), biopsy dishes and oil are placed in the incubator on day 2, allowing enough for each normally fertilized embryo to be biopsied in an individual dish.

On the morning of day 3, the embryos are assessed and their stage of cleavage and grade noted. A biopsy dish containing 2 × 10μl drops of biopsy media in a line just to the left of centre and a single 10μl drop on the right with an oil overlay is prepared for each embryo to be biopsied. Once the laser has been calibrated and the pipettes correctly positioned, the embryo is transferred to its corresponding biopsy dish washed through the upper drop, placed in the lower of the two drops and biopsied immediately. This ensures the exposure to the biopsy media is minimized (ideally no more than 5 minutes).

Figure 28.5 shows a cleavage stage biopsy using laser drilling.

The embryo is orientated on the holding pipette (e.g. Cook K-HPIP-1030) so the blastomere to be biopsied is at the 12 o'clock position. This allows a gap between adjacent blastomeres at the 1–2 o'clock position which can be used for drilling the zona, while minimizing heating of nearby blastomeres. On the Saturn Active Laser, Preset 1 is used for zona drilling. This gives a hole size of 5μm and a pulse width of approximately 0.254ms. If the zona is particularly thick, preset 2 is used. An opening of approximately 20μm is created which allows insertion of the biopsy pipette (Cook biopsy pipette K-EBPH-3030) with an inner diameter of either 30 or (K-EBPH-3535) with an inner diameter of 35μm. The blastomere is then removed by gentle aspiration. Depending on individual cells, the blastomere may be aspirated fully into the pipette and then removed, or partially aspirated and pulled out (this is the method we use most frequently). Aspiration of the blastomere for biopsy is the most

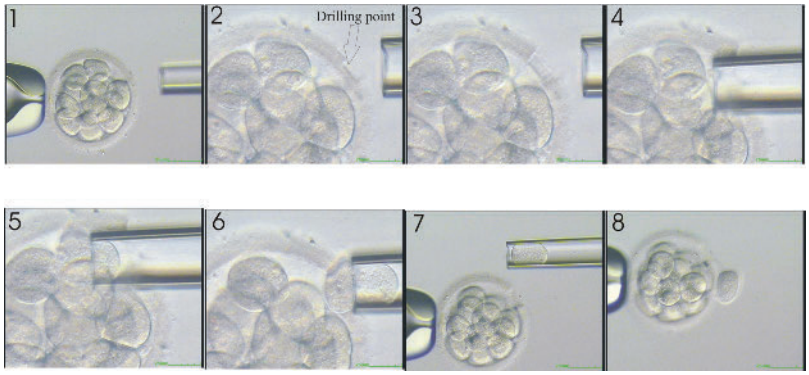

Figure 28.5 Laser assisted cleavage stage biopsy.

commonly used clinical technique worldwide. However, several other methods have been suggested:

The two main alternatives are 'extrusion', where the blastomere is squeezed through the hole in the zona pellucida by pushing against the embryo at a site below the opening. Levinson and colleagues [40] used acid drilling and extrusion for gender determination in clinical PGD. The second alternative is 'flow displacement' where a slit is made in the zona with a sharpened needle and then through a second puncture site media is injected into the embryo, dislodging a cell through the slit. This technique has had limited use in clinical PGD [41].

In our clinic we routinely only remove one cell, and the blastomere chosen should not be dividing and should have a clear nucleus. A second blastomere would only be removed if the first lyses. For single gene PGD cases if the cell lysis does occur, it is advisable to change the biopsy pipette before continuing to avoid any possible contamination with DNA from the lysed cell. If however the chromosomes are being tested using FISH, it is unlikely to cause a problem. Removal of two blastomeres significantly decreases the rate of blastocyst formation, as it depletes the embryonic mass by 12.5–25% or more and risks the inadvertent removal of critical cells [48]. Occasionally an embryo fails to show any nucleated blastomeres; in this case a cell is chosen which appears to best represent the embryo.

Multinucleation is also sometimes evident; these cells would be avoided when performing the biopsy as they have been correlated with an increased rate of aneuploidy, mosaicism and chromosomal abnormalities; a lower blastocyst formation rate and consequently a lower implantation rate [42–47]. These results suggest two possible patterns of multinucleation, one of which occurs at the 2-cell stage and produces chromosomally abnormal embryos. The other occurs at the 4–16 cell stage and usually involves bi-nucleation probably produced by cytokinesis failure, but each of the two nuclei is chromosomally normal. In true bi-nucleated cells both nuclei appear the same size and similar in dimensions to the single nuclei in other cells; these can then be treated like any other cell for biopsy. If however the nuclei are of varying size (i.e. micronuclei) they are best avoided. Embryos that have MNB at the 2-cell stage should not be recommended for embryo transfer, while embryos with bi-nucleated cells at the 8-cell stage may be considered if no other morphologically normal embryos are available. Multinucleation is also related to other morphological characteristics like fragmentation and cleavage rate. Not surprisingly, the lowest incidence is correlated with minimal fragmentation and optimal cleavage; embryos with uneven sized blastomeres show a much higher rate of multinucleation and aneuploidy [43].

Aneuploidy rates in human embryos produced by IVF have been proven to be very high [48]. The data collected from FISH and CGH analysis indicate as many as 70% of day-3 embryos have chromosomal abnormalities. This rises to 82% in women over 40 years old [49–51]. Follicular stimulation is one potential factor that may increase aneuploidy, as it leads to the recruitment of smaller follicles that would not be selected in a natural cycle and may already be predisposed to aneuploidy. However, this is only one possibility, and it is worth noting that data also indicate a high aneuploidy rate in unstimulated cycles.

After biopsy, further embryo development should not be impaired, with formation of morula or a least further cellular division being evident on day 4. In order to optimize blastocyst development our clinic

does not perform embryo checks on day 4; instead a full assessment is made on the morning of day 5. Interestingly, only one patient has failed to produce blastocysts after cleavage stage biopsy, all of her embryos appeared to have arrested prior to compaction. When the genetic results were obtained they all showed multiple chromosomal abnormalities. This is consistent with the work published by Sandalinas *et al.* [52], Clouston *et al.* [53] and Fragouli [54], who demonstrated blastocysts have a normal karyotype more commonly than cleavage stage embryos, indicative of a degree of selection against some of the most abnormal embryos.

It has been shown that comparable numbers of chromosomally abnormal cells are found in both the ICM and trophectoderm tissues [56]. The change from morula to blastocyst is critical in selecting against aneuploid cells. Evsikov and Verlinsky [57] observed that the degree of mosaicism up to the morula stage is much higher in comparison to that of the blastocyst. If the number of abnormal cells at the morula stage reaches a certain threshold, whole embryo degeneration is likely.

In order to offer more effective screening and exclude embryos which may arrest prior to blastocyst formation, our clinic has moved towards trophectoderm biopsy. Taking biopsies from the trophectoderm increases the number of cells available for analysis, as 2–9 cells are removed rather than only a single cell from a cleavage stage embryo. This provides more genetic material for analysis yet represents a lower proportion of the embryo's mass. It also avoids taking cells which may have been cleavage stage precursors of the inner cell mass, although whether cells truly have irreversible commitments at this time remains controversial.

Blastocyst trophectoderm biopsy

There are two possible approaches to breaching the zona to allow trophectoderm herniation. The first involves the zona pellucida being opened on day 3 of development, creating a 10–15μm opening using a 250ms pulse from the Saturn Active laser. The rationale behind a slightly smaller opening is that the use of the laser actually creates a trench in the zona rather than just a single hole allowing more cells to herniate anyway, but also it ensures the trophectoderm that herniates is thinner and therefore easier to remove.

Once skilled with the laser it is not necessary to position the embryo on a holding pipette for this procedure, which means it can be quickly performed in the day 3 culture dish prior to transfer to the blastocyst culture medium. The position at which the hole is to be created needs to be carefully selected to avoid potential damage to the blastomeres. Therefore it is important to only drill where there is adequate space either between blastomeres or where the largest area of perivitelline space is available if the embryo has compacted. Two holes are drilled adjacent to each other starting from the outer zonal membrane and progressing through the zona pellucida until the inner zonal layer is breached. The embryos are then kept in culture until day 5, when they are assessed for blastocyst formation. If suitable herniation has occurred (hopefully trophectoderm cells) the biopsy should be performed; if not the blastocysts are transferred to fresh blastocyst culture media, examined again in the afternoon of day 5 and the morning of day 6.

At the OFU we have found that drilling on day 3 has allowed a larger percentage of the available blastocysts to be biopsied in the morning of day 5, which has enabled fresh transfers to be performed on day 6 while maintaining good pregnancy rates.

A proportion of the blastocysts may herniate their inner cell mass. Two options are available; first the hole can be widened to allow more trophectoderm cells to be extruded, or second the blastocyst can be manually removed from the zona and the biopsy performed by gently rubbing off a section of trophectoderm cells.

Figure 28.6 shows an alternative approach, which involves making the hole at the blastocyst stage when the ICM is visible, the zonal breach being created directly opposite the ICM. For this procedure the blastocyst is secured on a holding pipette (Cook, internal diameter 15μm) and the zona very carefully drilled. After approximately 4 hours of culture, sufficient trophectoderm cells should be herniating to allow biopsy to proceed.

Figure 28.7 demonstrates the procedure for a naturally hatching blastocyst.

A biopsy dish (Falcon 1006) is prepared for each blastocyst to be biopsied and labelled with the patient details and blastocyst number. A line is drawn on the underside of the dish to mark the centre. On the left side of the line 2 × 20μl drops of Cook gamete buffer (Hepes) are pipetted and on the right side of the line

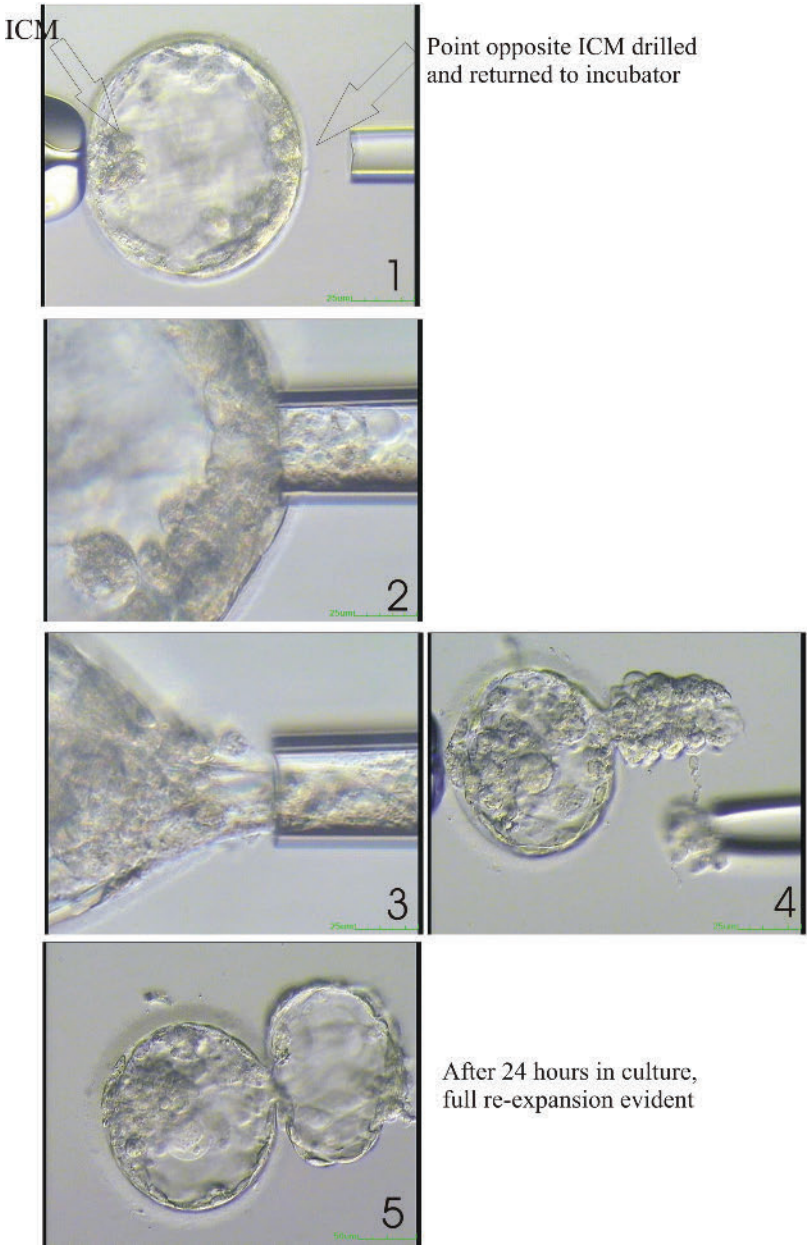

Figure 28.6 Laser assisted trophectoderm biopsy (drilled on day 5).

(in the lower position) 1 × 10µl drop of pvp (marked with a circle on the underside of the dish). 1 × 20µl drops of gamete buffer are added above the PVP; this is used for rinsing the pipettes if necessary during the procedure. This is covered quickly with approximately 4.5mls pre-equilibrated oil and the lid and then returned to the incubator until required.

In many cases the genetic analysis is sufficiently rapid that transfer in the afternoon of day 5 or in the morning of day 6 is possible. However if, through choice or necessity, cryopreservation is required, the blastocysts should be vitrified as soon as possible after the biopsy. Consequently, everything required should be prepared prior to commencing the biopsies.

A spare setup dish is also prepared to keep the pipettes primed during short breaks in the procedure. Obviously, to avoid contamination if longer breaks are necessary, new pipettes would be used.

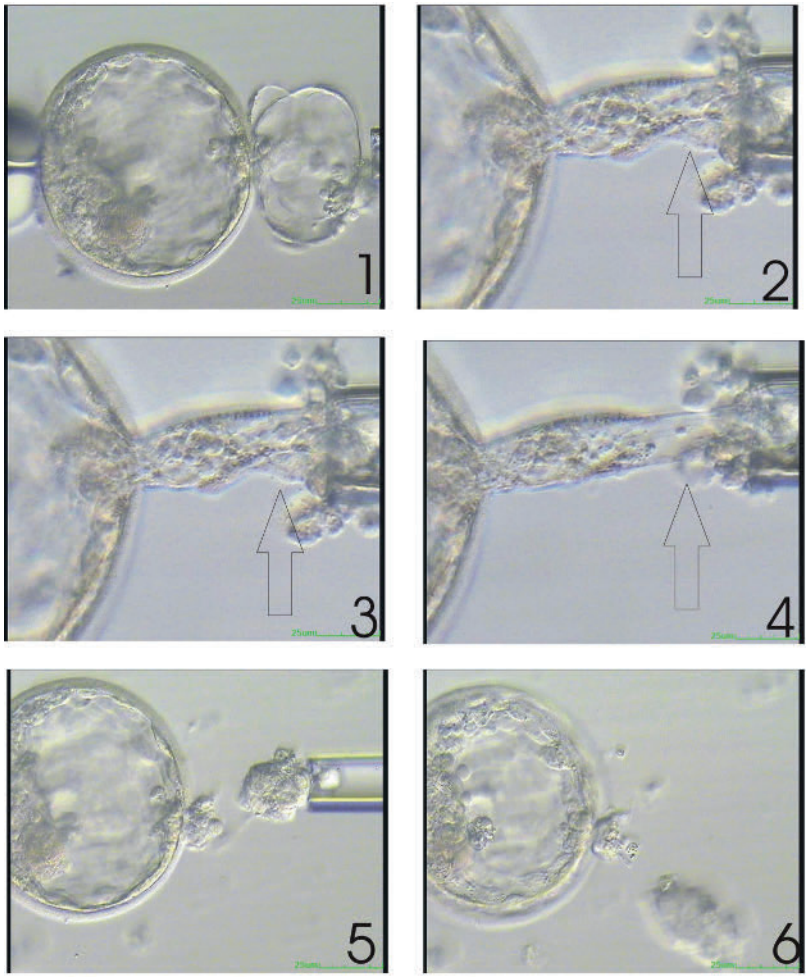

Figure 28.7 Laser assisted trophectoderm biopsy (natural herniation). Arrow indicates position of laser pulses.

A sterile, empty biopsy dish is used to set up the pipettes and position them just above the base of the dish. The number and quality of trophectoderm cells indicate the size of biopsy pipette that is required; the ideal size is usually 30μm but in some cases 35μm may be required. The pipettes are then primed just prior to use, the holding pipette is loaded with gamete buffer, while it is useful to coat the biopsy pipette with PVP as it prevents the biopsied tissue sticking.

In the presence of a witness the blastocyst to be biopsied is transferred to the corresponding labelled biopsy dish. It is placed in the top drop of gamete buffer on the left side of the line, as a wash step, and then transferred to the lower drop where it will be biopsied. The biopsy is performed by securing the blastocyst on the holding pipette with the herniating cells at the 3 o'clock position. It is important to ensure the blastocyst is touching the base of the dish; this ensures optimum laser efficiency. The biopsy pipette is placed close to the trophectoderm cells to be biopsied, and the cells are very gently aspirated into the pipette. Aim to take in 2–9 cells. As a rough guide, if the biopsy pipette is 30μm in diameter, aspirate approximately 30μm in length up the pipette. Care needs to be taken to avoid cell lysis, as amplification efficiency is significantly reduced if this occurs.

It is very important to ensure that the trophectoderm cells completely fill the mouth of the pipette; if media is seeping round the edges it could cause the biopsied portion to shoot up the pipette when it is separated. If necessary, it is best to change to a different sized pipette.

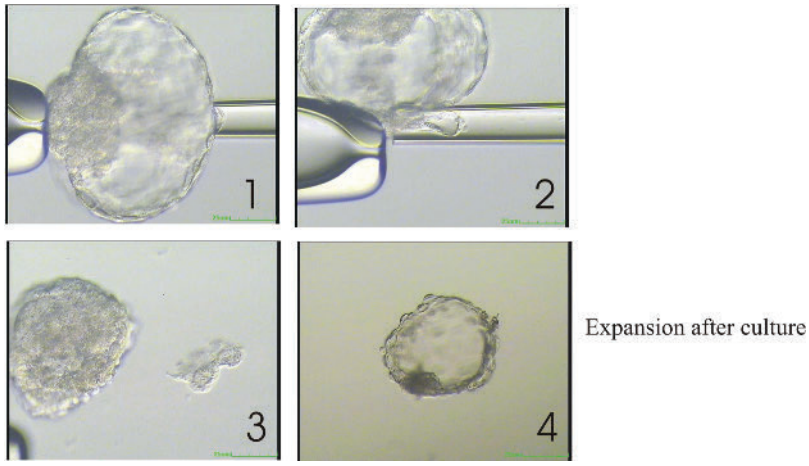

Figure 28.8 Trophectoderm biopsy of hatched blastocyst.

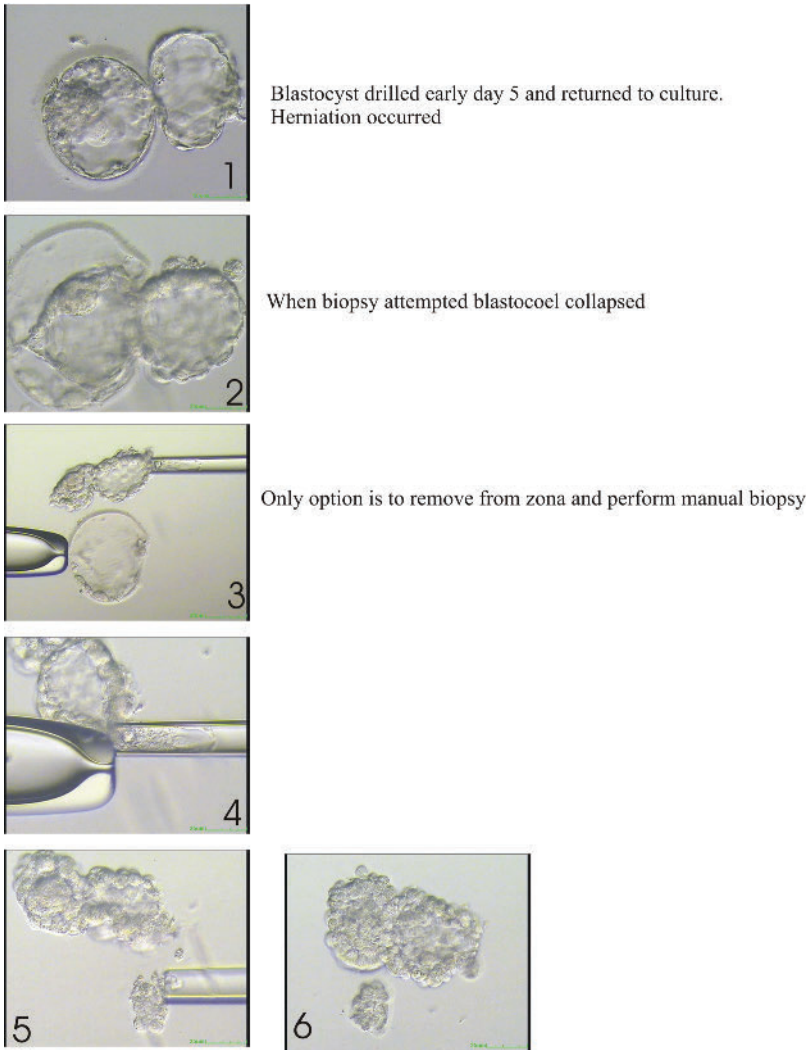

Figure 28.9 Manual trophectoderm biopsy after blastocyst collapse during laser biopsy.

The ideal pulse width for performing the biopsy is between 400–500ms; usually 3–5 laser pulses should be sufficient to detach the trophectoderm cells from the blastocyst. Aiming at the junction between the cells, fire one pulse at the top of the sample, one in the centre, then one at the bottom and repeat this sequence until the biopsy is complete. Moving up and down while firing ensures the cells don't become cauterized and hardened, making them impossible to separate. As the sample starts to move away from the embryo, remember to keep firing more to the right towards the biopsy pipette or the biopsy sample may become too large.

Mechanical pressure also plays an important role in separating the cells, so tension should be applied to both the holding and biopsy pipettes during the procedure. Once separate the biopsied material should be released into the drop away from the blastocyst. The blastocyst is witnessed back to its culture dish and the biopsied trophectoderm is then ready for sample preparation and transfer to a genetic testing service (e.g. Reprogenetics).

It is generally difficult to use the laser to slice off trophectoderm cells if the blastocyst has hatched, as it is not possible to apply the tension required on the area of the ICM directly. Therefore it is necessary to use mechanical methods. Figure 28.8 shows the steps involved. Occasionally during the biopsy the blastocoele cavity collapses so fully that it is not possible to apply the tension required to separate the cells using the laser. It is therefore necessary to perform a mechanical hatching and biopsy as shown in Figure 28.9.

Vitrification

Historically it has been shown that embryos that have an opening in their zona are more sensitive to the effects of cryopreservation using slow freezing protocols, with a reduced survival and development rate [58, 59]. Several studies have demonstrated vitrification to be a very effective method for the cryopreservation of blastocysts as long as the blastocoele cavity is collapsed prior to vitrification [60–62]; this makes it ideal for biopsied blastocysts as the blastocoel typically collapses during the biopsy process. At OFU all biopsied blastocysts are vitrified within 1 hour of biopsy before any re-expansion of the blastocoele has begun using CVM fibreplug and sleeve (Cryologic – Australia).

The success of a PGD/PGS programme depends on the experience and expertise of the embryologists performing the techniques, with embryo biopsy the single most important factor in achieving satisfactory outcomes after genetic testing.

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Chapter 29

In vitro maturation of oocytes

Gustavo German and Tim Child

Aim of chapter

The in vitro maturation (IVM) of oocytes has emerged as a promising assisted reproductive technology (ART) treatment. In this chapter, our goals are to: (1) describe the benefits of IVM compared to other treatments; (2) give an historical perspective; (3) provide the biological background required to understand the type, quality and quantity of oocytes available in women for IVM treatment; (4) describe the procedure as it is performed in our clinic; (5) discuss the evidence supporting current practices in IVM; (6) review the outcomes of IVM in comparison with routine in vitro fertilization (IVF) for different patient populations; (7) refer to the limited data on IVM obstetric and perinatal outcomes; and (8) examine the problems faced in IVM and the challenges to overcome.

ART as a successful clinical treatment for infertile couples

Although the first successful IVF procedures were performed within natural menstrual cycles, it was soon recognized that greater numbers of oocytes were required for the treatment to be efficient. Consequently, exogenous gonadotropins are now routinely used, in combination with gonadotropin-releasing hormone (GnRH) agonists or antagonists. The increased availability of oocytes, and therefore embryos, allows the selection of one or two high-quality embryos or blastocysts for uterine transfer. The most recent data from our clinic (the Oxford Fertility Unit) reports a 42% live birthrate per IVF/ICSI cycle started in women < 35 years of age. This means that cumulative IVF success rates are at least comparable to rates achieved during natural conception among fertile couples. However, the use of multiple embryo transfer and controlled ovarian hyperstimulation (COH) to achieve these excellent rates can result in complications: multiple pregnancy and ovarian hyperstimulation syndrome (OHSS).

Ovarian hyperstimulation syndrome

All forms of exogenous ovarian stimulation carry the risk of inducing OHSS, an iatrogenic and potentially lethal condition with a diverse range of symptoms. In its mildest form, OHSS involves symptoms such as weight gain, abdominal bloating and mild nausea, while the moderate form can include vomiting, shortness of breath and reduced urine output. Severe cases involve evidence of intravascular dehydration and fluid accumulation in the third space such as ascites and hydrothorax, along with hypovolemia, haemoconcentration, oliguria and electrolyte imbalances with potentially fatal consequences [1]. Risk factors include gonadotropin stimulation, young age (less than 35 years), a history of OHSS and ultrasound evidence of ovaries of a polycystic morphology (PCO) (defined on transvaginal scan as one containing 12 or more antral follicles of 2–9 mm diameter or increased ovarian volume (>10 cm^3) [2]). This increased risk remains regardless of whether or not there are additional manifestations of the full polycystic ovarian syndrome (PCOS) [3] such as irregular ovulations or hyperandrogenism (clinical and/or biochemical). Among women of reproductive age, about 20% show PCO on ultrasound scan, but only 5–10% are considered to be affected with PCOS [2]. Swanton and colleagues [3] reported that younger women with PCO or PCOS undergoing IVF had significantly higher rates of severe OHSS (12.6% and 15.4%, respectively) compared to those with normal ovaries (2.7%) [3]. The overall rate of severe OHSS in the IVF population is around 1%.

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

IVM as an alternative to IVF

Since the only way to avoid the development of OHSS is to avoid ovarian stimulation, there is increasing interest in the use of unstimulated IVM. Unfortunately, natural cycle IVF is associated with low success rates in single figures. Although, compared to other ART treatments, IVM is currently more labour-intensive in the laboratory, the absence of gonadotropin stimulation not only eliminates the risk of OHSS but also avoids some of the disadvantages associated with gonadotropin use such as high drug costs, daily injections and frequent monitoring (ultrasound scans and estradiol levels). In addition, the absence of the pituitary suppression achieved with GnRH-agonists in long-protocol IVF eliminates the distressing menopausal side effects of estrogen withdrawal.

Historical perspectives

During the 1930s Gregory Pincus and colleagues reported a series of experiments examining the ability of mammalian oocytes to mature and fertilize in vitro. In 1935, they described spontaneous nuclear maturation, that is, germinal vesicle breakdown and extrusion of the first polar body in rabbit immature oocytes excised from antral follicles and placed in various culture media. Their observations were extended in a 1939 report examining maturation in vitro of human oocytes from ovaries removed at operation. Robert Edwards extended Pincus's observations in reports during the 1960s. In one study, insemination of in vitro matured human oocytes was successfully attempted.

The first live birth resulting from immature oocytes retrieved from unstimulated ovaries, and matured and fertilized in vitro, was reported by Cha and colleagues in 1991. In this report, donated immature oocytes were obtained from ovaries removed at laparotomy for gynaecological indications. In 1994, Trounson's group in Australia reported the first successful attempts at obtaining immature oocytes using transvaginal ultrasound guided retrieval.

Availability of immature oocytes

Ovarian reserve

By the fifth month of gestation all fetal oogonia, the germ cell precursors, have entered meiosis and arrested at the diplotene stage of prophase I. At this point each oogonium has been transformed into a primary oocyte, surrounded by a few flattened somatic pre-granulosa cells, which together form the primordial follicle, situated in the ovarian cortex. Through atresia (apoptosis or programmed cell death) the finite stock of resting follicles decreases exponentially from mid-fetal life (7 million) until the menopause, which occurs when only approximately 1000 follicles remain. Independently of extra-ovarian control, some primordial follicles start to develop daily. Since only about 400 oocytes are ovulated throughout a woman's reproductive life, the vast majority of oocytes that were initially present are lost because of atresia, not ovulation.

While this manuscript was in press, Tilly and colleagues reported in *Nature Medicine* the striking discovery of a rare ovarian population of presumptive human oogonial stem cells (OSCs) with similar characteristics to the previously identified mouse OSCs [34, 35]. The mouse OSCs were shown to have the capacity to multiply in vitro and differentiate into functional and developmentally competent oocytes in vitro and in vivo. If this dogma-breaking discovery holds true, the potential for artificially extending the ovarian reserve and women's fertility is encouraging.

Follicular growth

There are three main stages of development en route to ovulation. The primordial follicle becomes a preantral follicle, then an antral follicle and finally a preovulatory follicle (Fig. 29.1). Initially, the granulosa cells divide and proliferate, forming a secondary follicle. After several granulosa cell layers have formed, a fluid-filled cavity, the antrum, appears and expands. This phase of growth is regulated by local interactions between the oocyte and somatic cells. Thereafter, follicular development is subject to endocrine control, predominantly FSH. In women with normal ovaries, from the recruitable pool of antral follicles (2–5 mm), about 7–14 follicles are selected to grow in the ovulatory wave of the menstrual cycle [4]. By definition, women with PCO have a greater number of 2–9 mm antral follicles. Usually only one follicle, the dominant follicle, will continue to grow more than 10 mm and eventually be ovulated. The rest of the follicles will undergo atresia. The LH surge signals the final stages of follicular/oocyte maturation prior to ovulation. One of its effects is stimulation of cumulus expansion (mucification), which occurs as a result of secretion of a hyaluronan-rich extracellular matrix by the granulosa cells surrounding the oocyte (cumulus cells). This matrix expands and keeps the cumulus cell-oocyte complex (COC) together, whose configuration is thought to facilitate oocyte extrusion at ovulation and its capture by

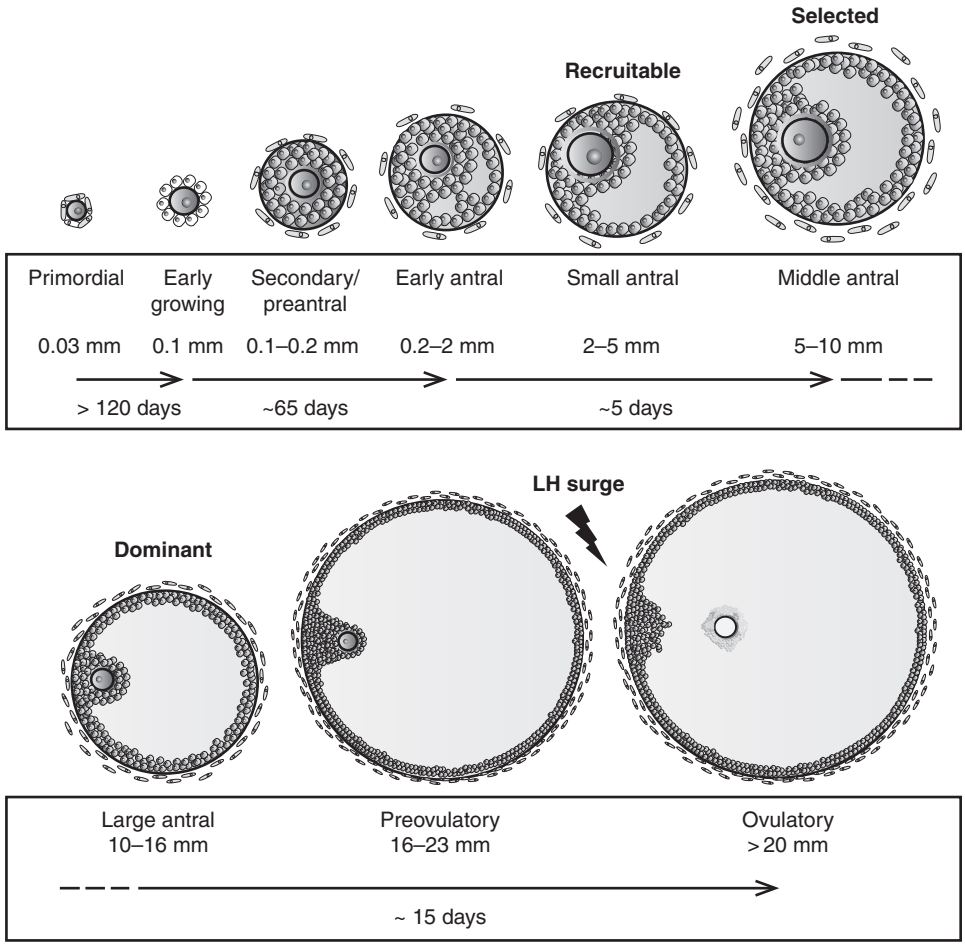

Figure 29.1 Follicular developmental stages in women. The diagram shows the progression of follicles from the primordial stage to the ovulatory stage (Figures not drawn to scale). The approximate follicular size at each stage, and the time required to progress from one stage to more advanced stages, are included. Beginning at 2–5 mm, follicles are FSH-dependent. Usually only one follicle per cycle develops further than 10 mm and becomes dominant, while the rest of the cohort of growing follicles undergo atresia. The LH surge signals the final stages of follicular/oocyte maturation. Adapted from Gougeon (1996) [5].

oviductal fimbria. In addition, the expanded cumulus plays a role in sperm penetration and fertilization.

The extent of oocyte maturation to be achieved in vitro

The aim of IVM is to induce normal oocyte maturation within the COCs (Fig. 29.2) retrieved from the small-middle antral follicle stage (2–10 mm diameter). In vivo, this process takes roughly 5–15 days and occurs within the follicle as it grows to the preovulatory follicle stage (16–20 mm diameter). With current IVM technologies, oocyte maturation is accelerated due to spontaneous meiotic progression. Developmentally competent oocytes can be obtained after 24–48 hours of culture in vitro after oocyte retrieval but not thereafter. Given that the growth period from the primordial follicle to the preovulatory follicle takes more than 6 months [5], IVM attempts to induce only the very last stages of oocyte maturation. Most of the oocyte growth has been achieved during the previous maturation stages where the oocyte increased its diameter from about 30 μm in the primordial follicle to its final diameter of 120 μm, representing a 65-fold increase in volume. The cytoplasmic growth is accompanied not only by the development of the zona pellucida and growth of cellular organelles but also by accumulation of large amounts of mRNA and protein, which are essential components to sustain early embryonic development. Despite the substantial

growth achieved, it is thought that the capacity of an oocyte to resume and complete meiosis and, after fertilization, develop into a blastocyst is acquired progressively during preovulatory development by increasing follicular size [6]. One of the major challenges of IVM is that of creating suitable conditions for this process to occur in vitro.

In humans, the follicle size at ovulation in the natural cycle is about 17–26 mm diameter. In conventional IVF cycles, matured MII oocytes are usually aspirated from 14–23 mm diameter follicles, while in the case of IVM cycles most COCs are retrieved from 2–10 mm follicles (Fig. 29.3).

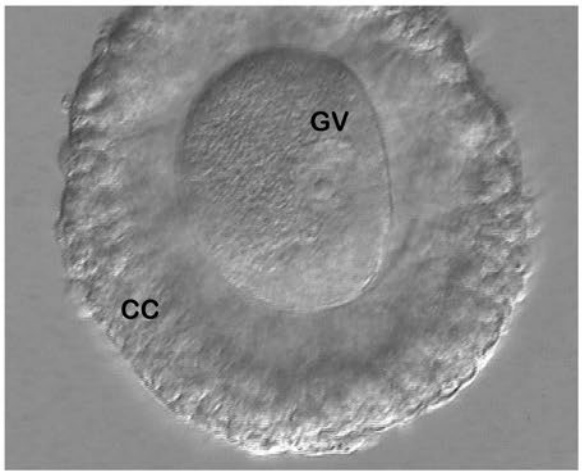

Figure 29.2 Picture of a COC retrieved from an unstimulated ovary. The germinal vesicle (GV) can be observed within the oocyte, which is surrounded by a compact mass of cumulus cells (CC).

Final oocyte maturation

A critical event occurring as a result of the LH surge is nuclear maturation – the progression of chromatin status from prophase I arrest to the metaphase II (MII) block. Three stages during this process can be identified microscopically, each determining the maturation status of the oocyte. First, the nuclear membrane is intact and the nucleus can be visualized. The oocyte is at the germinal vesicle (GV) stage (Fig. 29.4, GV). On resumption of meiosis, the nuclear membrane disintegrates. When the nucleus is no longer visible, the oocyte is at the germinal vesicle breakdown (GVBD) stage (Fig. 29.4, GVBD). At this point the cell is still in meiosis I and consequently is sometimes referred to as an MI oocyte (no nucleus, no polar body). The first meiotic division finally culminates in the extrusion of the first polar body (PB1) and is rapidly followed by the second meiotic division. Observation of the PB1 indicates the end of nuclear maturation, at which point the oocyte is described as an MII (Fig. 29.4, MII) and becomes ready for fertilization.

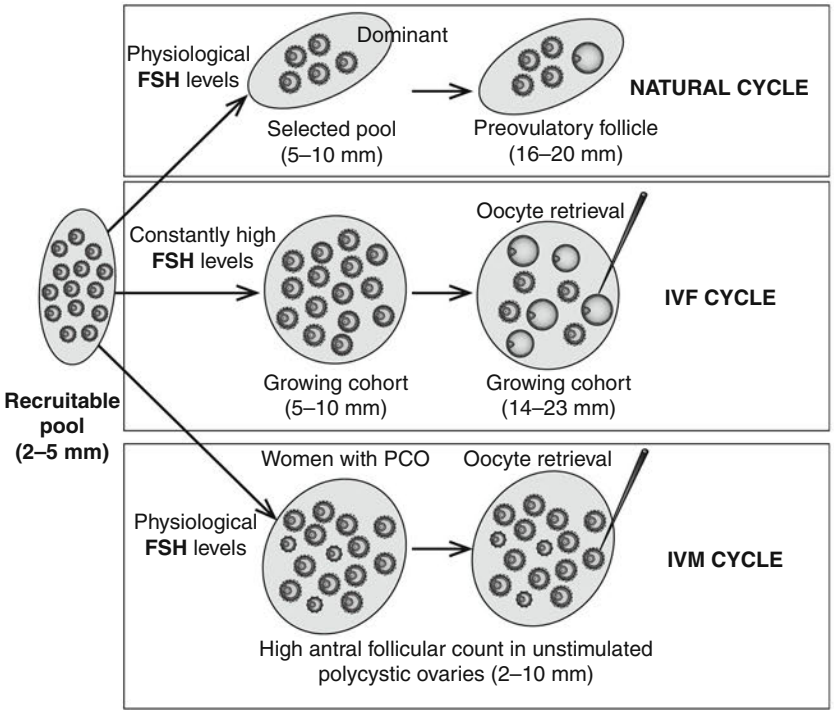

Figure 29.3 Comparison of the fate of the recruitable pool of follicles in the natural, IVF and IVM cycles.

In addition to nuclear maturation, the less defined process of cytoplasmic maturation is necessary for obtaining a developmentally competent oocyte. This process is primarily active during the preovulatory period prior to meiotic resumption. It involves remodelling of cellular organelles, accumulation of nutrients and other substrates, appropriate gene expression and modification of gene transcripts and proteins. In contrast to nuclear maturation, however, there are no methods during oocyte IVM to determine the state of cytoplasmic maturity without destroying the oocyte.

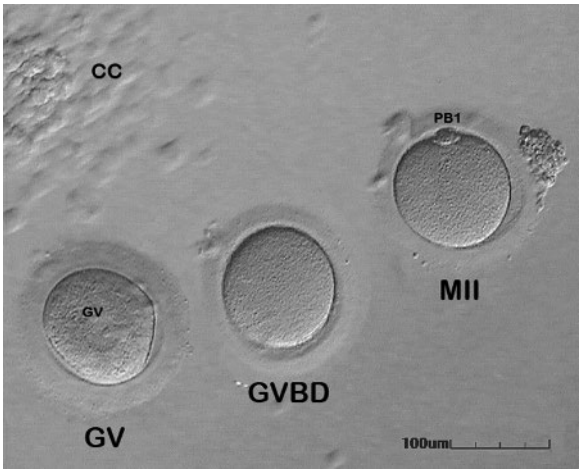

Figure 29.4 Stages of nuclear maturation. Pictures of oocytes at the GV, GVBD and MII stages. At the GV stage, the nuclear membrane is intact and the nucleus (GV) is visible under the microscope. Upon GVBD, neither a GV nor a PB1 can be observed. The MII oocyte shows the extruded PB1. On the top left corner, there is a clump of cumulus cells (CC).

Differences in treatment between IVF and IVM

There are a number of differences between IVF and IVM cycles. Primarily, IVF, unlike IVM, requires ovarian stimulation. In addition, during IVM, blood tests for monitoring estradiol levels are not necessary, oocytes are aspirated from smaller follicles and matured in vitro and the endometrial preparation includes estrogen supplementation in addition to progestagens (Fig. 29.5).

The protocol we use is heavily based on the McGill University, Montreal protocol [7]. Currently, infertile women with PCO aged less than 36 are eligible for IVM. A pretreatment ultrasound scan confirms that the ovaries contain sufficient antral follicles and are

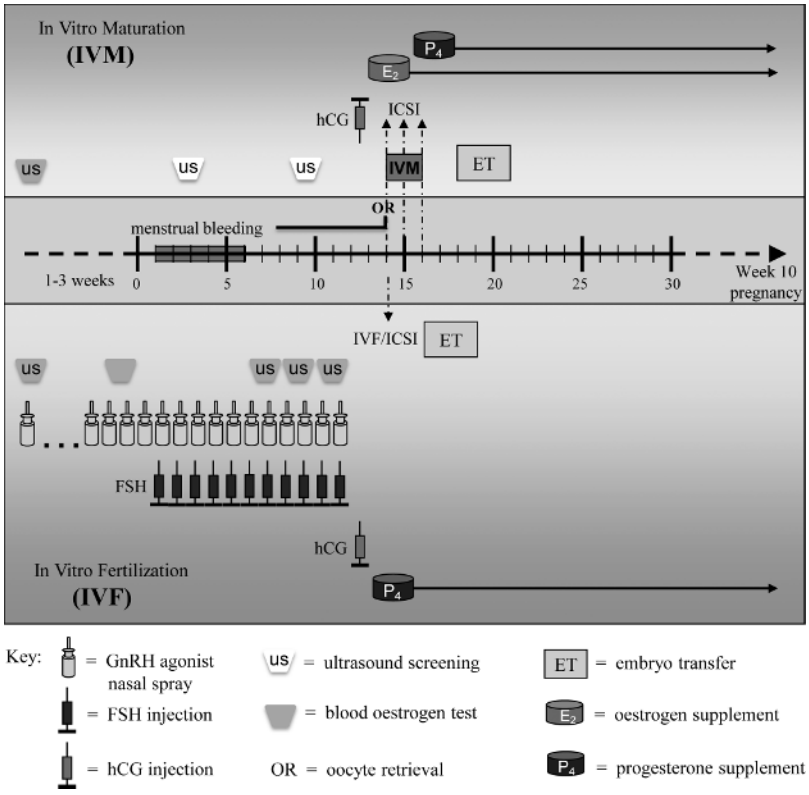

Figure 29.5 Timeline diagram comparing IVF (long protocol) with IVM.

accessible transvaginally. While ovaries stuck high in the pelvis due to endometriosis/adhesions may be accessed transvaginally during standard IVF when enlarged following gonadotropin stimulation, the same is not true for small, unstimulated ovaries.

For amenorrhoeic women a withdrawal bleed must be induced with oral progestagens. Patients call on day one of their menstrual cycle (either spontaneous or induced) and undergo a transvaginal ultrasound scan between days 2–5 to confirm the ovarian antral follicle count and accessibility, and absence of pathology such as corpus lutea cysts.

Anovulatory women are given a date for immature oocyte retrieval between days 7–14 of their spontaneous or progestagen-induced cycle. For ovulatory women we aim to perform the retrieval before there is a dominant follicle greater than 14 mm, so a second ultrasound scan may be performed to assist with timing. The only gonadotropin drug used is a single subcutaneous injection of hCG 10 000 IU, 35 hours prior to immature oocyte retrieval, which is the same regime used during standard stimulated IVF.

'Short-protocol' IVF using GnRH antagonists are increasingly used as an alternative to 'long-protocol' GnRH agonist cycles. While PCOS patients undergoing IVF are significantly less likely to develop severe OHSS with the short, rather than long, protocol, the risk will always exist [8]. There is no risk of OHSS during IVM.

The rationale behind IVM methods

Size of largest follicle at oocyte retrieval

For ovulatory women undergoing IVM, the timing of oocyte retrieval is important since it is understood that following the development of a dominant follicle, the remaining cohort of follicles undergo atresia. However, evidence suggests that the developmental competence of oocytes from small antral follicles persists, or is even improved by the presence of a dominant follicle [9]. While the mechanism of atresia in human follicles smaller than 1 mm is characterized by fast and early oocyte degeneration, oocyte integrity is maintained for longer in atretic follicles greater than 1 mm [5]. It has been suggested that the ideal time for collecting immature oocytes may be when the dominant follicle measures 12–14 mm diameter [9]. In addition, such a strategy is more likely to provide an in vivo matured MII oocyte from the dominant follicle in addition to a number of immature oocytes from the smaller follicles.

Type of insemination

Extended culture of oocytes produces hardening of the zona pellucida, which can decrease fertilization rates by IVF [10]. Almost all IVM cycles reported used ICSI as the method of fertilization, irrespective of the sperm parameters, in order to overcome the effects of zona hardening.

Gonadotropin priming

Ideally, patients undergoing IVM treatment do not need gonadotropin drugs. However, in women with PCOS, a single administration of 10 000 IU of hCG 36–38 hours before oocyte retrieval was demonstrated to improve the number of MII oocytes obtained at oocyte retrieval, oocyte maturation time and blastocyst formation [11]. To increase the number of good quality oocytes obtained during immature oocyte retrieval, several groups used mild ovarian stimulation with low doses of FSH during the early part of an IVM cycle. Such a strategy could prolong the midfollicular phase FSH level and decrease the rate of atresia on the antral follicular cohort. Two studies following this approach in women with PCO/PCOS obtained conflicting results [12, 13]. In women with normal ovaries, a recent RCT designed to evaluate the optimal gonadotropin priming protocol in 400 women found that priming with 150 IU of FSH/day for 3 days from day 3 of the cycle plus 10 000 IU of hCG 36–38 hours prior to oocyte retrieval yielded a significantly higher clinical pregnancy rate per transfer (29.9%) than non-primed cycles (15.3%, $P < 0.05$), hCG-only-primed cycles (7.6%, $P < 0.0001$) or FSH-only-primed cycles (17.3%, $P < 0.05$) [14]. Thus, it is apparent that the use of gonadotropins, FSH priming for some patients and hCG prior to oocyte retrieval for all patients, is beneficial during IVM treatments. Nonetheless, there is no need to suppress endogenous gonadotropin secretion (e.g. GnRHa), and the quantities of recombinant FSH, when used, are considerably lower compared with IVF, reducing the cost and patient burden during ART treatment. In addition, no cases of severe OHSS were reported with the low stimulation regimens during IVM treatment.

Endometrial preparation

During a stimulated IVF cycle, the endometrium is exposed to supraphysiological levels of endogenous estrogen. In a frozen embryo replacement cycle, however, the endometrium is prepared by administering estradiol valerate beginning in the early follicular phase. A RCT

showed that early exogenous estrogen administration during an IVM cycle was detrimental to the oocyte developmental potential [15]. Thus, it is a challenge to adequately prepare the uterus during an IVM cycle, in the few days between oocyte pick-up and embryo transfer [16]. Most reported IVM cycles utilized estradiol administration from oocyte retrieval. Accordingly, the endometrium exposure to estrogen is short in IVM compared to other ART treatments, a factor that may explain the reduced relative success rates of IVM.

Media composition

A culture media is designed with the aim of providing the oocyte with an appropriate environment that not only allows oocyte survival in vitro but also supports oocyte nuclear and cytoplasmic maturation. The first requirement is fulfilled by including energy substrates, amino acids, vitamins and buffering systems for pH, oxygen radicals, metal ions and osmolarity. To promote oocyte maturation, additives are included in the media such as growth factors, serum, gonadotropins and steroids. Studies to improve culture media for optimal human oocyte maturation are lacking due to the scarcity of research material. Most informative data come from animal studies. For IVM of human immature oocytes, complex culture media, developed originally to culture other cell types, such as TCM-199, Ham's-F10 and Chang's medium, have been adapted by the addition of various supplements [6]. Currently, most clinical IVM programmes use one of the two commercially available IVM culture media from CooperSurgical (SAGE IVM Kit, ART-1600) or Origio (MediCult IVM System, 8221). Both IVM media are designed to be supplemented with gonadotropins (FSH and LH/hCG). The MediCult IVM medium also requires addition of the patient's own serum. At the time of writing, no studies comparing SAGE IVM with MediCult IVM media have been reported.

Additives

Estradiol concentrations in human follicular fluid are significantly higher in follicles containing mature oocytes than immature oocytes, with increasing levels correlating with larger follicular size [17]. In addition, human oocytes contain mRNA for the estrogen receptor [18]. Thus, estrogen could have a physiological role during oocyte maturation and is therefore a candidate factor to include in the culture media. Since estradiol is produced during the culture of COCs, it has been suggested that its inclusion in culture media may not be necessary [7].

Serum is often used in somatic cell culture with beneficial effects. It is a source of protein and has heavy metal scavenger activities, in addition to other potential detoxification functions. The main beneficial constituents, however, are thought to reside in undefined growth factors, which promote growth. Addition of serum for embryo culture has been discouraged because of the potential transmission of infectious agents and other serum-induced trauma resulting in alterations in organelle ultastructure, metabolism, gene expression, genomic imprinting, and fetal development [19]. Serum could have similar negative effects on oocyte culture. Although the use of the patient's own serum avoids the risk of transmission of infectious agents absent in the mother, such a practice precludes standardization of culture conditions because the undefined factors present in serum are likely to vary between individual patients as they do between the different batches of animal sera. It has been common practice in clinical IVM programmes to use serum from animal origin (FBS) or patient's serum [20]. The use of human serum albumin (HSA) present in the formulation of SAGE IVM kits appears to be a good alternative to patients' serum [7]. Follicular fluid from large follicles has also been used as an alternative to serum to supplement culture media [20]. However, their use raises similar concerns as serum supplementation.

Although gonadotropins (FSH and LH) have well-known physiological roles in follicular development in vivo, their addition to culture media to improve oocyte maturation has resulted in contradictory results in human and animal studies [6]. Nonetheless, the majority of clinical reports include FSH and LH supplementation in their IVM culture media.

A number of growth factors have been proposed as supplements to improve maturation of immature oocytes during IVM culture. For example, epidermal growth factors (EGF), secreted by mural granulosa cells, mediate in a paracrine fashion the downstream effects of nuclear maturation initiated by the LH peak. Most published reports have not used EGF supplementation in the IVM culture media. Systematic studies of growth factor supplementation for IVM culture of human immature oocytes are lacking.

Time-length of oocyte in vitro culture

Most studies report in vitro culture of immature oocytes up to 48 hours. There is evidence that oocytes

undergoing nuclear maturation in 24 hours are developmentally more competent than oocytes maturing after 48 hours [21]. Also, it has been shown that oocytes reaching the MII stage at 24 hours have lower chromosomal abnormalities, leading to embryos with a lower aneuploidy rate [22]. We no longer culture immature oocytes (GV or GVBD) past the afternoon of the day after oocyte retrieval (30 hours culture). In our experience, oocytes requiring further culture have shown very poor developmental potential.

Predictive factors associated with treatment outcome

The main predictive factors associated with IVM treatment success are the number of oocytes retrieved, which is predicted by ultrasound measurement of the antral follicle count, and younger female age [23]. The success rate is proportional to the number of embryos produced [24]. During unstimulated IVM treatments, the average number of oocytes retrieved from women with normal ovaries is significantly lower than from women with PCO/PCOS.

Comparison of success rates between IVF and IVM

In order to evaluate the benefits of IVM treatment, it is necessary to consider its success rates compared with IVF in women with or without PCO.

IVM seems better for women with PCO/PCOS compared with normal ovaries

In an observational study, IVM outcomes in women with PCO, PCOS and normal ovaries were compared [25]. Significantly better implantation rates (8.9% PCO, 9.6% PCOS, 1.5% normal), pregnancy rates (23.1% PCO, 29.9% PCOS, 4.0% normal), and live birthrates (17.3% PCO, 14.9% PCOS, 2.0% normal) were found in the first two groups of women when compared with the third group. However, the group of women with normal ovaries was significantly older – a factor known to contribute negatively to the results.

Women with PCO/PCOS have similar pregnancy outcomes to women with normal ovaries after IVF

Swanton and colleagues [3] could not detect a significant difference in live birthrate per cycle among women with PCO (38%), PCOS (37%) and normal ovaries (40%) during IVF treatment [3]. Overall, in contrast to IVM, there seems to be no difference in IVF treatment outcomes between women with PCO/PCOS and women with normal ovaries.

Women with PCO/PCOS seem to have reduced outcomes with IVM compared with IVF

For women with PCOS, a recent Cochrane review attempted to compare live birthrates after IVM with IVF, but could not reach any conclusions due to the absence of RCTs [26]. Using the IVM methods described above, a retrospective case-control study, which is in preparation for publication, found a significantly lower live birthrate for IVM compared with IVF in women with PCO/PCOS (Table 29.1). However, the significantly higher rate of severe OHSS in the IVF group still makes IVM treatment an appealing choice for this patient population.

Patient population most likely to benefit from IVM treatment

In addition to women with PCO, IVM treatment has been suggested as an alternative to conventional IVF in other patient populations such as women who respond poorly to ovarian stimulation with exogenous gonadotropins and women who require fertility preservation, especially those whose impending chemotherapy treatment precludes delay, since cytotoxic agents may impair a woman's future fertility. Increasingly, natural-cycle IVF combined with IVM of immature oocytes (natural-cycle IVF/M) is sought as an alternative to COH for women with regular cycles, in the presence of various causes of infertility [27].

Obstetric and perinatal outcome of children born after IVM

Over 1000 IVM babies have been born worldwide with retrospective data on neonatal outcomes being collected by the Montreal group, led by R. C. Chian. The combined data were presented at ESHRE 2008 and suggest no obvious concerns. However, formal, prospective, paediatric follow-up studies are limited. Buckett and colleagues (2007) reported similar obstetric outcomes and congenital abnormality rates among babies born following IVM, IVF and ICSI [28].

Table 29.1 Outcome of IVM compared with IVF for women with PCO/PCOS undergoing treatment in the Oxford Fertility Unit

	IVM	IVF	
Patients (n)	125	125	
Mean age	32.8	32.8	
Cycles (n)	125	125	
Number oocytes retrieved (SD)	17.4 (9.6)	15.3 (7.4)	
Maturation rate	66%	N/A	
Implantation rate	14.2%	37.2%	P<0.05
Biochemical pregnancy rate	28.0%	59.2%	P<0.05
Clinical pregnancy rate	21.6%	52.0%	P<0.05
Live birthrate	18.4%	42.4%	P<0.05
Severe OHSS	0	11 (8.8%)	P<0.05

SD, standard deviation; N/A, not applicable.

Neurological development of IVM children at 2 years of age appears to be normal [29].

Factors explaining reduced IVM success rates

The two main factors that might explain the reduced success rates of IVM cycles are (i) decreased endometrial receptivity and (ii) reduced oocyte/embryo developmental potential.

Endometrial receptivity

A successful implantation requires a competent embryo and a receptive uterus. Using a mathematical model, Rogers *et al.* [30] estimated that endometrial receptivity contributed 31–64% to the probability of a successful implantation [30]. The period of maximal endometrial receptivity is called the implantation window, and in humans, it is thought to occur around days 20–24 of the natural cycle. The endometrium during IVM cycles is exposed to relatively low levels of estradiol by the time of oocyte retrieval. Exogenous estradiol administration from the day of oocyte retrieval is given with the hope of synchronizing the implantation window with embryo development.

Ultrasonography to measure endometrial thickness has been evaluated to predict endometrial receptivity during IVM. Child and colleagues [16] found that increasing endometrial thickness at the time of embryo transfer was correlated to higher implantation and clinical pregnancy rates up to a threshold of 10 mm, although the endometrial thickness at the time of immature oocyte retrieval was not predictive [16].

Developmental potential

The second factor thought to affect implantation rates in IVM cycles is oocyte quality. During an IVM cycle, retrieving COCs from 2–10 mm follicles creates the difficulty of re-creating in vitro the physiological intrafollicular environment required for normal oocyte maturation.

Oocyte maturation

As nuclear maturation occurs prematurely, it stops the process of cytoplasmic maturation, in which the oocyte acquires the capacity to support preimplantation development [31].

Nuclear maturation

The negative consequences of disrupted oocyte maturation are highlighted by evidence suggesting that oocytes matured in vitro have a significantly higher incidence of abnormalities of the meiotic spindle and chromosome configuration [32].

Cytoplasmic maturation

This term refers to the process of accumulation of mRNA, proteins, substrates and nutrients taking place during oocyte development [31]. One of the key events during oogenesis is the synthesis of RNA and protein products required to support early

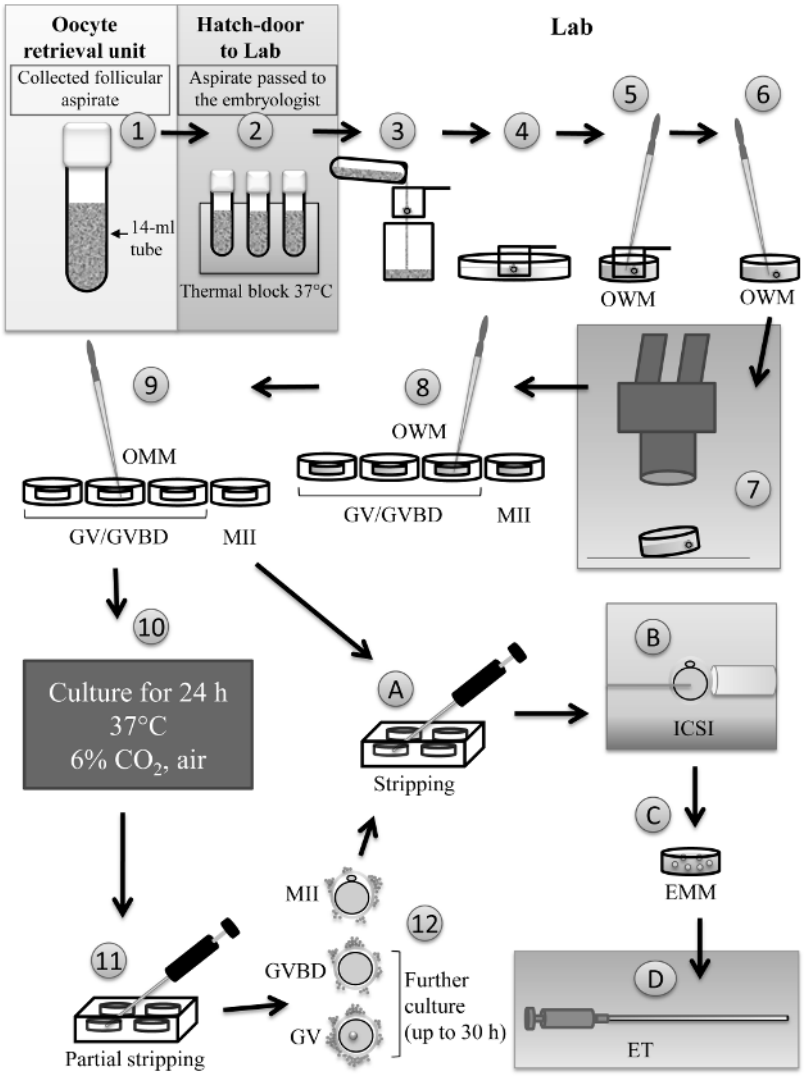

Figure 29.6 The follicular aspirates are filtered through a 70 µm nylon mesh (BD Falcon, Cell Strainer 352350) over a 60-ml pot (steps 1–3). Blood will pass through the filter but the COC will be retained. If required, heparinized flush medium may be poured through the filter to clean it. The bottom of the filter may also be cleaned by briefly placing it in a tissue culture dish (BD Falcon, 60×15 mm) containing heparinized flush medium (step 4). The filter is then moved to a tissue culture dish (BD Falcon, 35×10 mm) containing 3ml of Oocyte Washing Medium (OWM) (step 5). The dish with filter can then be placed back in the incubator until the next step in order to process other follicular aspirates. The tissue is later transferred with a pipette to an identical dish (step 6). The OWM comes buffered with HEPES to prevent significant changes in pH during the procedure. Every clump of cells is examined for COCs. Oocytes are identified and their nuclear maturation status assessed using the sliding method, which consists of sliding the COCs down the dish while observing under a stereomicroscope (step 7). While not in use, filters are placed in a resting dish containing OWM. Filters and tissue culture dishes are rotated throughout the procedure to ensure they are not out of the incubator for excessive lengths of time. Once a COC is identified, it is moved to an organ culture dish (BD Falcon, 60×15 mm) containing 1ml of Oocyte Washing Media in the centre well covered with 1ml of oil (step 8). Oocytes at MII, if found, are placed in separate dishes from GV and GVBD oocytes. After all aspirates have been examined, COCs are transferred to organ culture dishes containing 1ml of Oocyte Maturation Medium (OMM) supplemented with 75 mIU of FSH and 75 mIU of LH in the centre well and 1.5ml in the outer well, washing the COCs in the outer well briefly before placing them in the inner well (step 9). Oocytes are re-assessed for maturity 1–2 hours following oocyte retrieval, and any MII oocytes are prepared for ICSI (step A). Immature oocytes are cultured for maturation (37ºC, 6% CO_2, air and 100% humidity), until 24–27 hours post-oocyte retrieval (step 10). After 24–27 hours culture, all oocytes are partially stripped with hyaluronidase (ICSI Cumulase, Origio), using a 140 µm inner diameter capillary pipette, to assess the nuclear maturation status (step 11). If MII oocytes are identified at any stage of the procedure (on the day of oocyte retrieval, or after 24–30 hours culture), they are prepared for ICSI 1–2 hours in advance, by stripping the cumulus cell from the oocyte aided with hyaluronidase, using a 130 µm inner diameter capillary pipette (steps A-B). Following ICSI, oocytes are transferred into a 50 µl droplet of Embryo Maintenance Medium (EMM), after washing them twice in the same medium, and cultured overnight (step C). Immature oocytes are kept in culture and re-assessed later in the day (step 12). MII oocytes are prepared for ICSI while the rest will be discarded at the end of the procedure. Fertilization checks should be performed as per standard protocol. It is not necessary to transfer embryos to another medium, and so culture to embryo transfer (ET) is performed in EMM (steps C-D). Finally, standard protocols for embryo assessment and embryo cryopreservation should be utilized.

embryonic development until zygotic genomic activation at the 4–8 cell stage. Previous work has shown that maternal mRNA synthesis and storage occurs during follicular growth and ceases before GVBD [31]. Suboptimal IVM culture conditions could have a negative impact on oocyte maturation by altering the normal pattern of gene expression [33].

In an attempt to perform more physiological in vitro oocyte maturation, Gilchrist and colleagues devised a sequence of maturation steps in order to extend the time prior to and during nuclear maturation, providing an opportunity for further cytoplasmic maturation [36]. The rationale was based on accumulated knowledge on the cross-talk between oocyte and follicle cells and the factors controlling meiotic arrest and progression [37]. By manipulating these factors using meiotic inhibitors and activators, embryo (mouse and bovine) and fetal yields (mouse) were improved compared with conventional IVM [36]. Research on such an in vitro culture regime in human oocytes and its safety is required, but the prospects for an improvement in the clinic over traditional maturation methods seem promising. Until in vitro culture conditions are optimized, IVM is unlikely to be able to match the pregnancy rates achieved using conventional IVF. With current methods, in vitro matured oocytes do not have the same developmental competence as those matured in vivo. As well as chromosomal imbalance, imperfect coordination of nuclear and cytoplasmic maturation may contribute to the insufficiency of IVM oocytes.

Summary points

IVM appears an attractive treatment option for certain patient populations, especially for women at high risk of developing OHSS.

Secondary benefits from IVM treatment include absence of side effects from hormonal stimulation (menopausal symptoms and mild OHSS), lower drug costs, reduced ultrasound scans and blood samples, resulting in a simpler and less invasive treatment for the patient.

Immature oocyte retrieval requires special adaptations (narrower needle, lower pump pressure, frequent flushing, multiple punctures and additional sedation) in order to recover the COCs from the smaller follicles in the unstimulated ovaries.

IVM treatment is more demanding in the laboratory than routine IVF.

IVM outcomes are dependent on the number of immature oocytes obtained at oocyte retrieval which is best predicted by the antral follicular count.

Women with PCO have better IVM success rates than women with normal ovaries.

Disregarding OHSS risks, women with PCO have better pregnancy outcomes with conventional IVF than with IVM.

Oocytes retrieved from small-middle antral follicles can spontaneously undergo nuclear maturation in vitro.

Nuclear maturation halts cytoplasmic maturation.

With current culture conditions, oocytes matured in vitro have a decreased developmental potential compared with in vivo matured oocytes.

A deficiency in endometrial receptivity, due to the abbreviated estrogen exposure, is another potential factor thought to negatively impact IVM outcomes.

Future clinical and research efforts are needed in order for IVM to reach success rates comparable to those of IVF. Such an achievement could revolutionize the practice of ART, making it potentially less expensive, and certainly less invasive and much safer.

The IVM laboratory procedure as performed in the Oxford Fertility Unit can be seen in Figure 29.6.

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Chapter

30

Morphological expressions of human egg and embryo quality

Mina Alikani

Introduction

A critical step in the complex process of human in vitro fertilization (IVF) and embryo transfer (ET) is embryo selection. Selection is based on 'quality' which refers primarily to the gross morphology of embryos as it is known to pertain to viability. Whether morphology is a reliable marker for viability and implantation potential remains the subject of vigorous debate. Here, we present the view that morphology is not simply about aesthetics, even if embryologists often describe morphologically normal embryos as *beautiful*. Morphology is a reflection of biology, but not every morphological aspect is clinically significant and the correlation between morphology and clinical outcome is often not absolute. Thus as a sole (subjective) reference point for embryo selection, morphology falls short of ideal in accuracy and reliability.

Intense efforts have been underway for some time to transform embryo quality assessment into an objective and quantitative method. At the present time the only routinely applied quantitative methods are preimplantation genetic diagnosis and screening (PGD/PGS – see Chapters 33 and 34) which allow detection of genetic and numeric or structural chromosome abnormalities in oocytes and embryos. Several other methods, collectively referred to as 'OMICS' and considered to be minimally invasive are under investigation [1]. These methods involve measuring proteins (proteome) and other factors (secretome) released by the embryo into the culture medium (proteomics), products of metabolism (metabolome) in spent culture media (metabolomics), or gene expression through measurement of RNA content (transcriptome) in eggs, cumulus/granulosa cells or embryos (transcriptomics). Additionally, measurement of oocyte respiration rate [2] as well as egg and embryo mitochondrial activity [3] have been proposed as potential methods for quality assessment. Undoubtedly, the next few years will see a more comprehensive assessment of these technologies in the clinical laboratory, and hopefully, the development of an integrated approach to embryo quality assessment that encompasses morphology but addresses its shortcomings.

The focus of this chapter is on the most common and, in our view, the most clinically relevant morphological abnormalities observed by simple microscopic examination of human oocytes and embryos in the course of culture in vitro. These abnormalities result from developmental disturbances that fall into several categories, but are – for the most part – oocyte-related. They include disturbances in: oocyte maturation, chromosome segregation during metaphase and anaphase of meiosis I and II, cytokinesis of meiosis I and II, chromosome segregation during metaphase and anaphase of early mitoses, cytokinesis of early mitoses, karyokinesis and early differentiation. Such disturbances are strongly correlated with development potential and should be considered during embryo selection for intrauterine transfer or cryopreservation for subsequent attempts at pregnancy.

The oocyte

Following the luteinizing hormone (LH) surge (in a natural cycle) or human chorionic gonadotrophin (hCG) administration (in a stimulated cycle), human oocytes resume the process of nuclear (and cytoplasmic) maturation during a period of about 36 hours. Release from the diplotene stage of prophase I and progression through the remaining stages of meiosis I (MI) is morphologically evidenced by germinal vesicle breakdown (GVBD) and formation of the first polar body. At the subcellular level, the diploid chromosome content of the oocyte (2n; 4c; where 'n' is the number of chromosomes and 'c' is the DNA content

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

represented in chromatid number) is halved, with the oocyte and the first polar body each arriving at a haploid state while the DNA content remains double (1n; 2c). DNA content is reduced in half following fertilization (1n; 1c) when a second polar body is extruded. Chromosomes within the first polar body are associated with remnants of microtubules, while in the oocyte the chromosomes are aligned at the centre of a bipolar spindle, attached to the microtubules.

At ovulation (or follicular aspiration), the mature egg is arrested in the metaphase of meiosis II (MII) with the meiotic spindle located in proximity of the first polar body, close to and perpendicular to the oolemma. There is no interphase between telophase I and metaphase II in the oocyte, so a nucleus does not become visible. The maturation stages of the human oocyte assessed by transmission electron microscopy are depicted in Figure 30.1.

Apart from a single fully formed polar body, the meioticly mature, morphologically normal human egg has an intact, translucent zona pellucida, which is formed during the preantral phase of folliculogenesis. The cytoplasm of the normal egg is (mostly) uniform and is (mostly) free of inclusions. A distinct area of granularity is visible in a majority of eggs, although this does not appear at a fixed position; on occasion, there are two areas of granularity, or the area covers the entire egg circumference. This granular area represents a polarized distribution of mitochondria and has implications for cytoplasmic maturity [4]; its complete absence is negatively correlated with survival and normal fertilization following intracytoplasmic sperm injection (ICSI). Periodic and circular waves of granularity have been noted following fertilization and the duration of the cytoplasmic wave has been correlated with embryo quality [5]. It is not entirely clear but it is very likely that this post-fertilization granularity wave begins at the area of granularity that appears in normal eggs prior to fertilization. This may explain the observation concerning poor outcome following ICSI in eggs without a granular area. A relatively common abnormality is centrally clustered organelles/central granularity (a bull's eye appearance). Although this morphology appears to negatively impact fertilization rate, its impact on clinical outcome has not been conclusively demonstrated.

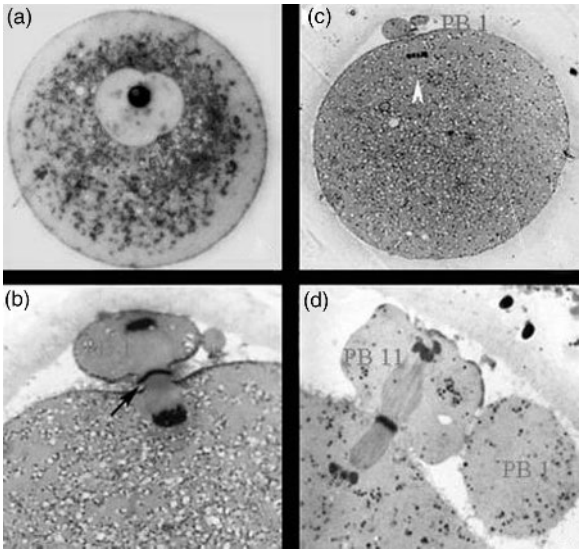

Figure 30.1 Transmission electron micrographs of human oocytes at four stages of maturation. (a) Germinal vesicle (GV) oocyte with one prominent nucleolus. (b) Oocyte at telophase of meiosis I, with the meiotic midbody (arrow) and the first polar body (PB1) visible. (c) Metaphase II oocyte, with the meiotic spindle (arrowhead) and the first polar body (PB1) visible. (d) Oocyte in telophase of meiosis II, with the first polar body (PB1) and the second polar body (PB11) visible. The chromosomes are visible at spindle poles. Original magnification x 400 (a and c) and x1000 (b and d). Micrographs are courtesy of Dr Henry Sathananthan, Monash University, Melbourne, Australia.

Meiotic maturation is also associated with changes in the egg vestments, partly under the influence of the oocyte itself [6]. The follicular cells surrounding a mature egg (cumulus cells) have undergone expansion or mucification, which entails enlargement of intracellular spaces, and the corona radiata cells are loosely arranged as a layer immediately surrounding the zona pellucida. Prior to GVBD, cumulus cells are linked with the oocyte through transzonal projections that reach and penetrate the oolemma. Interaction between the oocyte and follicular somatic cells is bidirectional [6].

The normal oocyte is roughly 120μm in diameter. The zona pellucida is approximately 17–20μm thick, but thickness does vary among patients and even among oocytes in a single cohort. The average zona pellucida diameter is inversely correlated with maternal age, possibly indicating diminishing oocyte/follicular activity during oocyte growth.

Nuclear immaturity is evidenced by presence of a GV (prophase of meiosis I). The cumulus appears clumped and the mass is generally small. Oocytes at the GV stage obtained following a clear LH surge after ovarian stimulation can be matured in vitro and they can be fertilized; however, they develop poorly and frequently fail to implant [7]. GV-stage oocytes obtained after initial ovarian stimulation without an induced LH-surge and retrieved from smaller antral

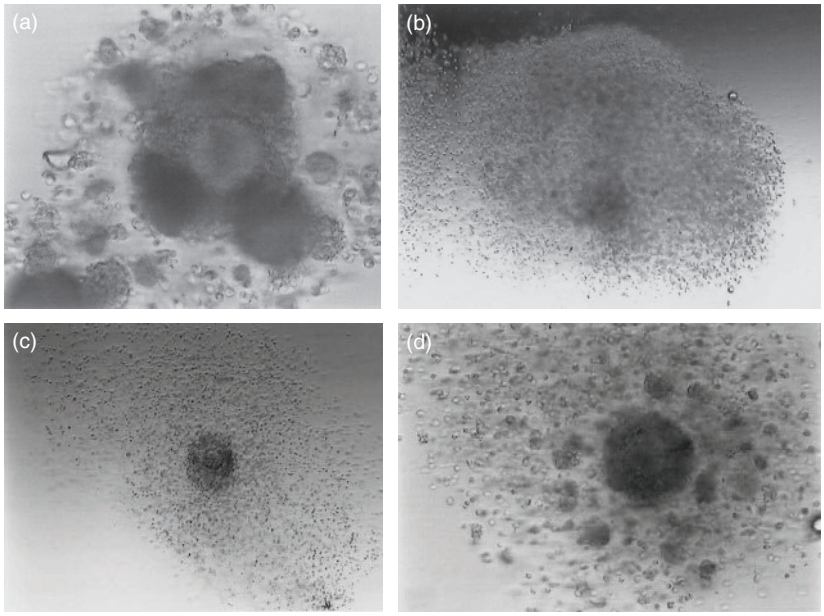

Figure 30.2 Oocytes retrieved from stimulated ovaries, showing different cumulus-corona complex (CCC) morphologies and nuclear maturity. CCC morphology can vary with different stimulation regimens and it can be discordant with nuclear maturity. (a) An immature GV stage oocyte; (b) an immature MI oocyte; (c) a mature MII oocyte; note the expanded cumulus mass; (d) a post-mature oocyte; note the clumped cells in the cumulus mass. Micrographs are courtesy of Dr G. John Garrisi, Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, NJ, USA.

follicles can be fertilized successfully and the embryos derived can develop fully [8]. The absence of a GV and the first polar body in gonadotropin-exposed oocytes is also an indication of nuclear immaturity (i.e. the oocyte is still in MI). The cumulus mass surrounding MI oocytes is not easily distinguished. Such MI oocytes can be matured in vitro and fertilized, but again, their development potential is reduced compared to eggs that are fully mature at the time of retrieval from follicles [7]. Post-maturity may be predicted by appearance of multiple cell clumps in the cumulus mass. Representative oocyte-cumulus-corona complex morphologies are depicted in Figure 30.2.

Unusually large oocytes, that is, those with a diameter of roughly 200μm or more, are abnormal. These so-called giant eggs occur rarely (roughly one in 1000) possibly as a result of errors in mitosis during proliferation of the oogonia. Giant eggs have been karyotyped and shown to be diploid; they can be fertilized but the resulting embryos are uniformly triploid, triploid mosaic, or polyploid [9, 10]. Thus these eggs should not be clinically used. The presence of a giant egg in a cohort of eggs is not associated with overall decreased pregnancy or implantation rate, but the resulting cohort of embryos may show an increased incidence of cleavage rate abnormalities [11].

An unusually large perivitelline space or reduced egg diameter (<108μm) is also considered abnormal and it is negatively correlated with development potential. It has been suggested that a large PVS is a sign of postmaturity of the oocyte. Some atypical first polar body shapes may be associated with oocyte abnormalities. Unusually large polar bodies may indicate disturbances in the position of the meiotic spindle, which may occur during oocyte ageing in vitro (and in vivo). Although 'fragmentation' of the first polar body has been identified as an abnormality in a number of publications, a thorough analysis of data obtained following polar body and blastomere biopsy suggests otherwise [12]. It should be noted that the first polar body can undergo spontaneous activation and division thus the description of polar bodies as 'fragmented' is not always accurate.

Other atypical features concern cytoplasmic organelles in mature eggs. The egg has many of the same organelles as somatic cells, including mitochondria (the most abundant), lysosomes, Golgi complexes and smooth endoplasmic reticulum (SER). Rough endoplasmic reticulum (RER) is not found in eggs [13]. The cortex of the mature egg is rich with cortical granules, arranged beneath the oolemma in discontinuous layers. At the light microscopic level, aggregation/hypertrophy of SER and accumulation of vacuoles are considered to be abnormal; these features are associated with oocyte ageing and represent degenerative processes in oocytes [13] and are associated with low development potential.

Using a Polscope, a microscope that makes use of polarized light to reveal highly ordered structures, the status of the meiotic spindle may be determined in denuded oocytes. Absence of the spindle at the time of sperm injection has been linked to reduced development potential. However, this state may be transitional (i.e. between telophase I and metaphase II) and there appears to be significant discrepancy between the Polscope images of the spindle (spindle birefringence) and the actual organization of its microtubules and chromosome components [14]. This is to say that a normal-appearing spindle on the Polscope is not necessarily normal when examined in detail by other means. Therefore the value of spindle imaging by polarized light in oocyte quality assessment is limited.

Representative micrographs of normal and dysmorphic eggs are shown in Figure 30.3. In summary, although numerous studies suggest a relationship between oocyte morphology and development potential, many of the specific findings are conflicting and ultimately difficult to interpret. In our view, it is questionable whether incorporation of egg morphology assessments per se in embryo selection schemes adds to the success of the latter. Nonetheless, these features are important and should be carefully noted for two reasons: first, it is clear that occurrence in one and the same egg of multiple atypical features is associated with reduced viability [15, 4], and second, unexpectedly high frequency of dysmorphic eggs in any given IVF cycle signals a potential problem in ovarian stimulation which may be corrected in a subsequent treatment cycle should pregnancy fail.

The zygote

Successful fertilization of an egg by a spermatozoon can be visualized as early as four hours post insemination. The normally fertilized egg displays two fully formed pronuclei with multiple visible nucleoli, two polar bodies (the second having formed as a result of completion of the second meiosis), a narrow but visible area of cortical clearing and an intact, translucent zona pellucida.

Clearly, the status of nucleoli or nuclear precursor bodies (NPB) within the male and female pronuclei is also of biological significance. Protein components of nucleoli play an important role in cell proliferation, primarily through regulation of ribosome biosynthesis and maturation [16]. Moreover, the polarization of chromatin content of pronuclei may be important in embryonic axis formation. During normal progression of nuclear events, nucleoli in each pronucleus move to one pole, some having coalesced, and they eventually align longitudinally where the pronuclei become apposed.

In a seminal time-lapse photographic study of the progression of fertilization events in the human egg

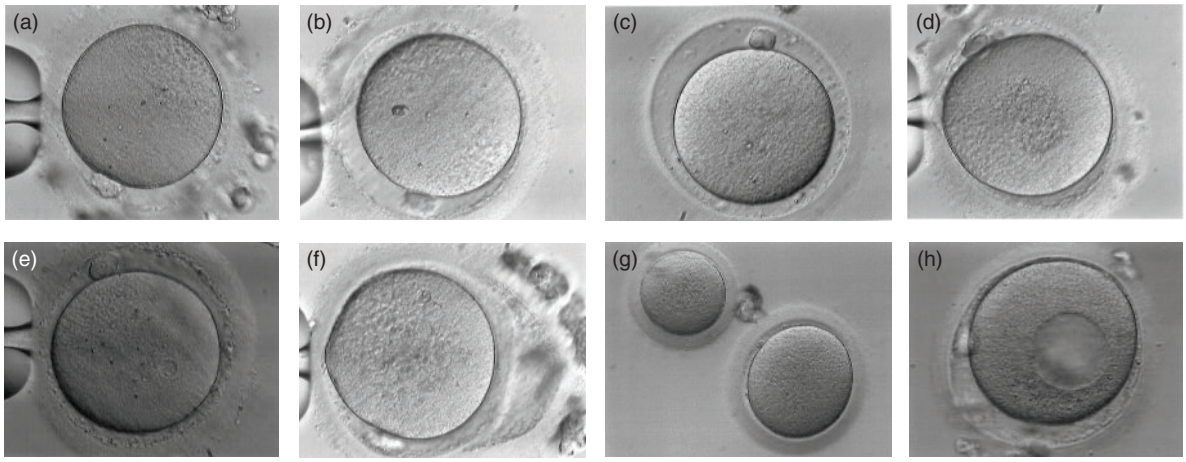

Figure 30.3 Morphologically normal and dysmorphic oocytes retrieved from stimulated ovaries. (a) MII egg with granular area at 1 o'clock and PB1 at 7 o'clock. (b) MII egg with two granular areas at 5 and 11 o'clock, and an inclusion (probably lipid) in the 9 o'clock position. (c) MII egg with a large perivitelline space. (d) MII egg with clustered organelles in the centre. (e) MII egg with presumed areas of necrosis and perivitelline debris. (f) MII egg with some central granularity and an abnormal multi-layered zona pellucida. (g) An abnormally large oocyte compared to a normally sized one. (h) MII egg with large vacuole or SER. Micrographs are courtesy of Dr G. John Garrisi, Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, NJ, USA.

following ICSI, Payne *et al.* [5] offered the following description:

> *Normal fertilization followed a defined course of events, although the timing of these events varied markedly between oocytes . . . there were circular waves of granulation within the ooplasm which had a periodicity of 20–53 min. The sperm head decondensed during this granulation phase. The second polar body was then extruded, and this was followed by the central formation of the male pronucleus. The female pronucleus formed in the cytoplasm adjacent to the second polar body at the same time as, or slightly after, the male pronucleus, and was subsequently drawn towards the male pronucleus until the two abutted. Both pronuclei then increased in size, the nucleoli moved around within the pronuclei and some nucleoli coalesced. During pronuclear growth, the organelles contracted from the cortex towards the centre of the oocyte, leaving a clear cortical zone. The oocyte decreased in diameter from 112 to 106 μm . . . during the course of the observation period.*

Fertilization or activation failure is evidenced by the absence of pronuclei and presence of only one polar body (i.e. failure of meiosis resumption); polyspermy is evidenced by the presence of more than two pronuclei and the presence of two polar bodies; digyny is evidenced by the presence of three pronuclei in the absence of a second polar body, the third pronucleus having resulted from failure of cytokinesis of MII and retention of the second polar body; sub- or micronucleation is evidenced by the presence of one or more small nuclei (subnuclei or micronuclei) in addition to the pronuclei.

Failure of the two pronuclei to become completely apposed is an abnormality, as is significant size discrepancy (>4μm) between the male and female pronuclei [17]. A higher incidence of complex chromosome abnormalities has been detected in embryos that result from zygotes with other atypical pronuclear morphologies [18]. The presence of small scattered nucleoli within pronuclei or overt discrepancy between the pronuclei with respect to position, size and number of nucleoli have been associated with a marked decrease in the number of euploid embryos (<10% were euploid) as well as a significant reduction in development potential [18]. On occasion, zygotes in which two pronuclei have been identified will appear to be single or tri-pronucleated at a later time before syngamy; embryos resulting from such single pronuclear configurations have morphological abnormalities and following transfer lead to very poor clinical outcome [19].

Other zygote features that have been assessed include the position of the second polar body in relation to the first, as well as the position of the polar bodies in relation to axis of the pronuclei. Although some relationships have been established, the ultimate value of such highly detailed zygote assessments may be limited. First, the assessment itself can be counterproductive in that the advantages gained by the information may be negated by prolonged exposure of zygotes to environmental stresses (e.g. fluctuations in temperature and pH)(J. Cohen *et al.*, unpublished observations). Second, nucleolar status changes with time in culture and categorization of zygotes at fixed time points may be misleading. Third, the frequency with which some deleterious patterns occur is low and the patterns are correlated with embryo morphology later during development. We are therefore led to conclude that unless transfer or cryopreservation is to be performed on day 1 of development, zygote morphology assessment should be restricted to key features discussed here (see Table 30.2). Indeed, other investigators have reached a similar conclusion using an empirical approach to build a predictive model for day-3 embryo selection [20].

Representative morphologically normal and abnormal zygotes are depicted in Figure 30.4.

The cleavage stages

On day 2 and day 3 after follicular aspiration, the morphologically normal embryo has four (day 2) to eight (day 3), uniformly sized, single nucleated blastomeres. The main abnormalities are alterations in cleavage rate and timing including retardation (asynchrony) of division affecting one or more cells, fragmentation, bi-nucleation (two equal-sized nuclei) in one or more blastomeres, sub- or micronucleation (a single nucleus chromosome content dispersed in multiple nuclei of varied size), and multinucleation (several nuclei, each with a full chromosome complement).

Alterations in cleavage rate and timing

A recent time lapse image analysis study [21] suggests that three early cleavage parameters before embryonic genome activation determine blastocyst formation in culture with >93% sensitivity and specificity: duration of the first cytokinesis defined as 'the very brief last

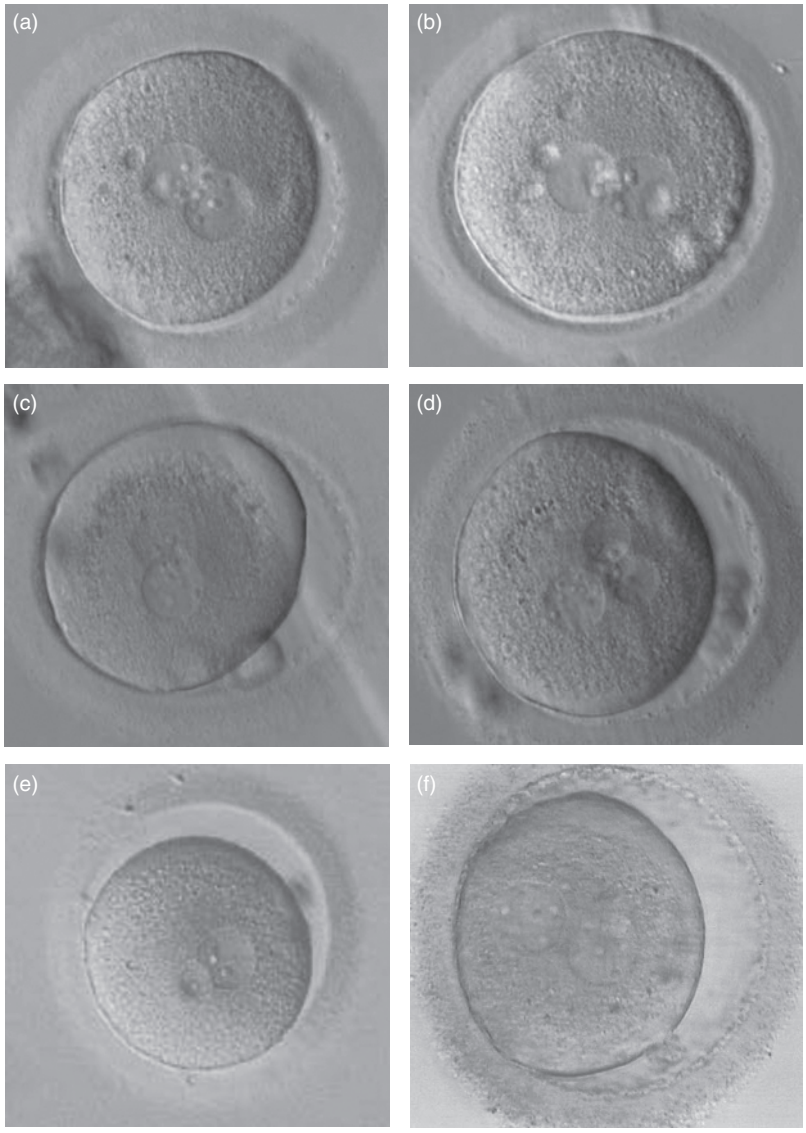

Figure 30.4 Fertilized human eggs. (a–c) Two pronuclei and small vacuoles (a and b). Two polar bodies are visible in c. (d) One normally sized pronucleus and two smaller pronuclei and a 'fragmented polar body'. (e) Unevenly sized pronuclei. (f) Separate pronuclei. Micrographs a–d were kindly provided by Dr Klaus Wiemer.

step in mitosis that physically separates the two daughter cells', time interval between the end of the first mitosis and the initiation of the second mitosis, and the time interval between the second and third mitoses (or 'appearance of the cleavage furrows of second and third mitoses') [21]. According to this study, those time intervals are 14.3±6.0 minutes (range of 0–33 minutes), 11.2±2.2 hours (range of 7.8–14.3 hours) and 1.0±1.6 hours (range of 0–5.8 hours), respectively. Since this study was done using frozen-thawed zygotes obtained from a single laboratory, it is not yet clear whether the specific timing observed by the investigators can be universally applied to fresh embryos, those grown under other culture conditions or those obtained from a different set of patients. Furthermore, this type of detailed morphological assessment will not be possible without the use of time-lapse imaging equipment. Nonetheless, it can be concluded that division timing and duration are both important determinants of development potential, as has been established in all animal models studied so far (Gonzales *et al*., 1995).

Division of the zygote into three cells suggests an abnormal tripolar spindle instead of a bipolar

structure. Tripolar spindles have been associated with polyspermic fertilization; this is probably a result of more than one centrosome (of male gamete origin) being present in the egg. But, tripolar spindles can conceivably form under other circumstances as well. Therefore equally sized 3-cell embryos should be excluded from transfer. Similarly, equally sized 6-cell embryos should be considered potentially abnormal.

It was recently hypothesized that completion of chromosome segregation in a given cell cycle in human embryos would vary depending on chromosome number [22]. Accordingly, embryos with mosaicism would be expected to show asynchronous division while euploid embryos would divide synchronously. A study was conducted to examine this hypothesis and it showed that 80% of the asynchronously dividing embryos were aneuploid, as assessed by FISH, while 53% of euploid embryos divided synchronously. Furthermore, the probability of implantation of day 3 embryos was highest for those with 8 cells; the rate was reduced for 6-cell embryos and was lowest for those with 12 cells [22]. Embryos with uneven cell numbers and those with >10 cells have been shown to have increased chromosome abnormalities [23].

It is generally agreed that embryos with fewer than 7 cells on day 3 (approximately 72 hours post-insemination) of development in culture are dividing more slowly than normal. Furthermore, a failure to divide in a 24-hour period is considered to be abnormal and may indicate development arrest. Slow cleavage is associated with increased chromosome abnormality [23], reduced blastocyst formation in culture and significantly reduced implantation potential [24, 25]. Data obtained from elective single embryo transfers suggest that delayed first mitosis on day-1–2 of development is an indication of a lower quality embryo as opposed to division within 25–27 hours of insemination or ICSI [26].

Fragmentation

Fragmentation is a common anomaly in human embryos. Precisely timed experiments in enucleated mouse eggs have shown that fragmentation occurs in the cytokinetic phase of the cell cycle [27]. The prerequisite to fragmentation is activation; unactivated eggs neither divide nor fragment, but fragmentation can be induced in activated eggs during both meiotic and mitotic divisions [27]. These experiments and others [28] also suggest that fragmentation occurs as a result of cytoskeletal disorder. Recent time-lapse studies in human embryos seem to confirm that fragmentation represents abnormal cytokinesis also in the human [21].

An inverse relationship has been shown between follicular fluid levels of cholesterol component, high-density lipoprotein (HDL), and fragmentation, leading to the suggestion that intrafollicular cholesterol metabolism may have an impact on oocyte membrane composition and structure, and in turn, the propensity of resulting embryos to fragment [29].

Fragmentation occurs to different degrees and in different patterns [30, 31]. Fragmentation occurs to different degrees and may show different patterns, although these parameters are subjectively evaluated. Accumulated data suggest that minor fragmentation affecting some 5–10% of the embryo is inconsequential to normal development. However, when it exceeds this level, a clear reduction in implantation potential is seen – the higher the degree of fragmentation, the lower the implantation rate until the probability of full development becomes virtually nil when fragmentation exceeds 50% [29].

This negative influence may be mediated through a number of mechanisms [29] including disrupted mitotic spindles, reduced cell number, reduced cell size, partial or total loss of regulatory proteins, disruption of cell interactions and signalling mechanisms, fewer mitochondria, abnormal mitochondrial distribution, low ATP content, failure of normal compaction and blastulation, cell allocation discrepancy and hatching failure. A summary of biological and clinical correlations of fragmentation is presented in Figure 30.5 and supportive references can be found in reference [29].

Fragmentation patterns have been defined based on size and distribution of the fragments relative to those of the blastomeres: type 1 with few small fragments mostly associated with one blastomere; type 2, many small fragments localized in one area, associated with one or more cells; type 3 with small and scattered fragments associated with most blastomeres; and type 4 with large and scattered fragments associated with a number of unevenly sized cells. Certain fragment types, for example types 2 and 4, show significantly fewer cells than other types [29].

Unequally sized blastomeres

Blastomeres may appear uneven because of loss of cytoplasm to fragmentation (for example in type 4

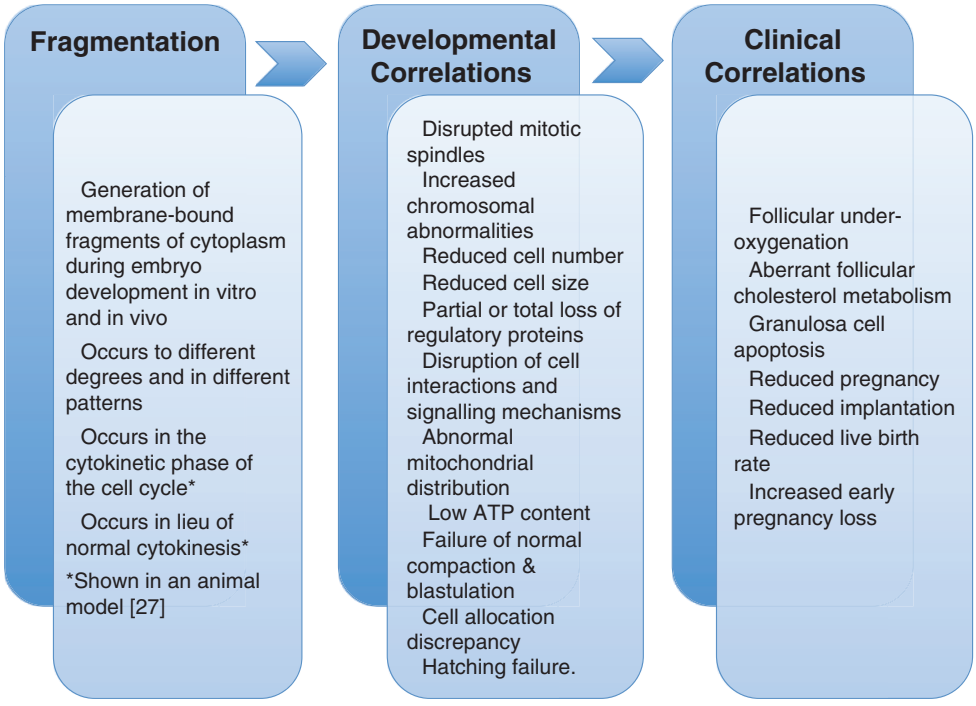

Figure 30.5 Human embryo fragmentation and its developmental and clinical correlations. Reprinted with slight modification from [29].

fragmentation), mitotic arrest in one or more cells at different stages of development, abnormal positioning of the mitotic spindle or asynchronous division. Whatever the cause, significant unevenness is associated with multinucleation, increased chromosome abnormalities and reduced development potential [30, 32, 25, 21].

Multinucleation

A normally dividing blastomere in a human embryo has a single visible nucleus unless it is in mitosis, in which case it appears anucleate (but appropriately sized with respect to the stage of development). At least one embryo with multinucleation is found in more than half of all IVF treatment cycles and some 17% of all embryos show this anomaly during in vitro culture [33]. Multinucleation can result from failure of cytokinesis following telophase of mitosis; it could also result from incomplete nuclear and cytoplasmic maturation [33]. Experimental evidence suggests that abnormal spindle formation and modified cytokinetic activity can lead to multinucleation in some cases (M. Alikani, unpublished observations).

Other factors have been investigated as potentially contributing to multinucleation, including maternal age, response to ovarian stimulation and culture conditions. Although a strict relationship between maternal age and multinucleation has not been established, it appears that the pattern of response in young and older patients may influence multinucleation. Patients with a high response to exogenous gonadotrophins as well as those who require a high dosage of gonadotrophins are more likely to have embryos affected with multinucleation. Moreover, modification of stimulation protocols appears to be effective in lowering the incidence of multinucleation in repeat cycles [33].

Three types of multinucleation may be distinguished in one or more blastomeres of day-2 or day-3 embryos: bi-nucleation where the two nuclei are equally sized, sub-nucleation where small nuclei appear along with one normally sized nucleus, and multinucleation where three or more nuclei are seen in each blastomere [34, 33]. It should be noted that a majority of studies do not distinguish among the different types of multinucleation.

Embryos with multinucleated blastomeres often result from zygotes with abnormal pronuclear

patterns; they have fewer and unequally sized cells and often a high degree of fragmentation. Significantly higher rates of polyploidy and extensive mosaicism have been detected in embryos with multinucleated blastomeres compared to those without multinucleation [33]. Somewhat expectedly, multinucleation has significant negative implications for blastulation in vitro, as well as pregnancy and implantation following intra uterine transfer. However, the latter correlation is nuanced in that some types of multinucleation, namely binucleation, may be less detrimental to clinical outcome than others and the developmental stage at which multinucleation occurs may be an additional predictor of clinical outcome, with day-3 multinucleation being more detrimental [33].

Abnormal zona pellucida

The zona pellucida is a protein-carbohydrate moiety that surrounds the mammalian egg and remains a crucial component of the embryo until implantation. This structure can appear dark or pigmented, multilayered, or distorted. The exact origin of these characteristics is not known and in most cases, their relationship to embryo viability is difficult to assess. However, zona pellucida thickness (ZPT) is an exception; both ZPT and ZPT variation (ZPTV) are predictive of embryo hatching ability, hence potentially also predictive of viability following in vitro culture [35]. ZPT decreases during in vitro culture and these changes are more evident in embryos that result in successful pregnancies. There is a correlation between ZPT and embryo quality (cell number and fragmentation) and maternal age. Increasing cell number as well as increasing maternal age (most notably >35 years) correlate with decreased ZPT, while increased fragmentation leads to decreased thinning rate and a thicker zona pellucida [36].

Introducing a gap (or a hole) in the zona pellucida by mechanical or chemical means or by the laser facilitates hatching and is one way to mitigate potential adverse effects of zona pellucida abnormalities. The procedure is also applied to embryos with cleavage abnormalities such as fragmentation. Whether assisted hatching leads to increased live birthrates is still debated, but it is clear that it leads to increased pregnancy and implantation. The procedure is more effective in certain patients, such as those with repeated pregnancy failure, and following embryo freezing and thawing.

Morula and blastocyst stages

On day 4 of development in vitro, a normally developing embryo becomes 'compacted'. Compaction is prerequisite to blastulation and involves a marked increase in adhesion between cells. A normally compacting embryo on day 4 of development shows a flattening of cells and the disappearance of cell boundaries.

The exclusion of multiple cells from the compacted mass of cells, previously referred to as partial compaction, is abnormal as is 'fusion-like' compaction in which the cell boundaries disappear completely. These abnormalities often lead to failure of blastulation [24].

On day 5 or day 6 of development, a normally developing embryo should form a blastocyst, with a single blastocoelic cavity, a distinct inner cell mass (ICM) and a coherent trophectoderm, both layers composed of many cells. A morphological grading system has been developed for blastocysts. It is quite useful since it communicates a detailed description of the ICM, TE and state of blastocoel expansion and has been shown to correlate with pregnancy and implantation [37]. According to this system, a numerical score from 1–6 is assigned to the degree of blastocoel expansion, referring respectively to: early blastocyst (1), blastocyst (2), full blastocyst (3), expanded blastocyst (4), hatching blastocyst (5), and hatched blastocyst (6). Blastocoel volume is given a score between 1 and 6 and described, respectively as occupying less than half, half, or the full volume of the embryo (without zona thinning); for scores 4–6, the blastocoel is described as larger than the volume of the early embryo (with a thinned zona). A score of 5 is given to blastocysts in which the TE has herniated through the zona pellucida, and a score of 6 indicates a fully hatched blastocyst. To describe the ICM, a letter from A to C is assigned respectively to: many tightly packed cells (A), several loosely grouped cells (B), and very few cells (C). Likewise, to describe the TE, a letter from A to C is assigned respectively to: many cells forming a cohesive epithelium (A), few cells forming a loose epithelium (B), and very few large cells (C). Thus, in this system, each embryo on day 5 and 6 of development is assigned a score that

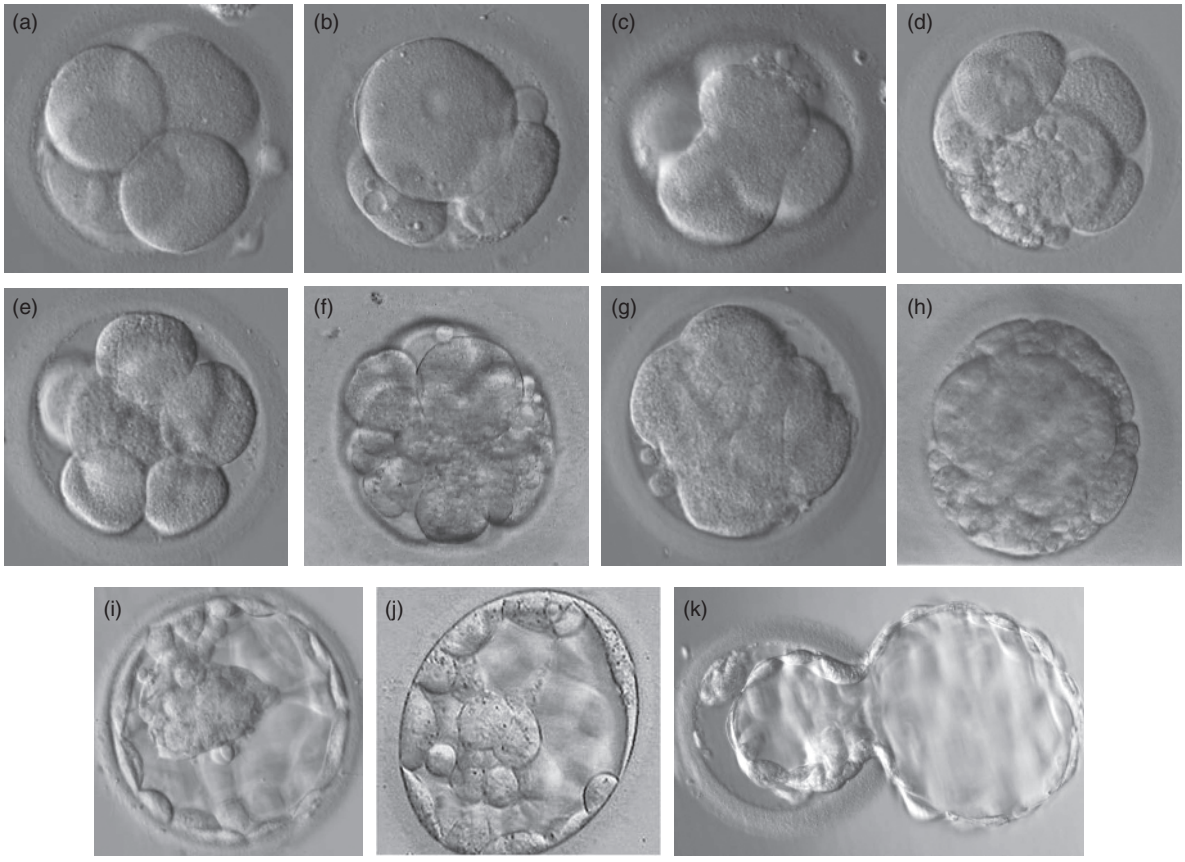

Figure 30.6 Morphologically normal and abnormal embryos on days 2 (a–d), 3 (e,f), 4 (g,h), 5 (i,j) and 6 (k) of development in vitro. Embryos depicted in a, e, g, l and k are morphologically normal. (b) Embryo depicted here has one dominant cell and two smaller cells all of which appear to be multinucleated. (c) This embryo shows signs of cytokinesis (elongation of the cell), however, there are multiple nuclei present in the 'dividing' cell; nuclei are not visible in a cell in mitosis, thus this is probably an abnormal cell. (d) A five-cell embryo with roughly 20% fragmentation, localized in one area; a small cell/large fragment is also present. (f) A day-3 embryo with many large fragments associated with unevenly sized cells. (h) A compacted embryo with many excluded fragments/cells. (j) A blastocyst with very few cells, particularly in the ICM. Micrographs a–e, g, l and k were kindly provided by Dr Klaus Wiemer.

consists of a number (1–6) and two letters (A–C), the best scores being equivalent to or greater than 3AA [37].

The most important abnormality on day-5 or day-6 embryos is the absence of an ICM, since the ICM gives rise to the fetus following implantation while the TE is the source of extra-fetal tissues. Low scores in the above system also indicate reduced implantation potential. Transfer of two embryos with one having a score of ≥3AA led to an implantation rate of 57% compared to an implantation rate of 33% when one of the two had a score of <3AA.

Representative cleavage stage embryos and blastocysts with normal and abnormal morphology are depicted in Figure 30.6.

Summary and conclusions

A summary of the points discussed in this chapter is presented in Tables 30.1 and 30.2. In this final section, we attempt to address the question of whether all or some of the parameters noted in this chapter should be assessed for the purpose of embryo selection. It appears that oocyte morphology is most useful for assessment of ovarian stimulation regimens. Large numbers of oocytes with cytoplasmic/extracytoplasmic abnormalities along with pregnancy failure indicate that changes in the stimulation regimen are advisable. On the other hand, very few oocyte morphological traits would definitively lead to their exclusion from transfer following fertilization and cleavage, unless those abnormalities (or others) are

Table 30.1 Key (and common) morphological features on different days of development in culture and whether the affected embryos should be excluded from transfer

Development Day	Morphology	Exclude from Transfer	Consider Excluding from Transfer[1]
0	Multiple Cytoplasmic Abnormalities		✓
1	PN Separate	✓	
1	PN Size Uneven	✓	
1	<2PN	✓[2]	
1	>2PN	✓	
2	1- or 3-Cell	✓[3]	
2	Multi-, bi-, micro-Nucleation		✓
2	>35% Fragmentation	✓	
3	5- or 6-cell or >10-cell		✓
3	Multi-, bi-, micro-Nucleation		✓
3	>35% Fragmentation		✓
4	No Compaction		✓
4	Abnormal Compaction		✓
5–6	Failed Blastulation	✓	
5–6	No ICM	✓	

[1] The reader is reminded that very few morphological features are absolutely correlated with failure of implantation; however, rather than relying on anecdotal instances in which some embryos with these anomalies do survive, the authors suggest a conservative leaning toward their exclusion from transfer based on (1) the majority that fails to implant and (2) the unknown consequences of adaptation for survival.
[2] Single pronucleate eggs obtained following ICSI should be excluded while those obtained following standard insemination may be left in culture and considered for transfer if they develop normally. Eggs that fail to show pronuclei on day 1 should be excluded from transfer; the author does not advocate re-insemination.
[3] Data overwhelmingly suggest that equally sized 3-cell embryos are chromosomally abnormal because of a tripolar spindle.

also present in the resulting embryos. The exception may be embryos resulting from oocytes with multiple abnormalities, as some studies suggest an increased incidence of early pregnancy loss following transfer of embryos obtained from such oocytes. On day 1 of development, it is crucial to assess pronuclear and polar body numbers but the value of other details may be questioned unless transfer or cryopreservation is to be performed on the same day. The exceptions here are those zygotes with overt pronuclear size discrepancy and those in which the pronuclei remain separate. During cleavage stages, division timing, cell number, fragmentation, synchronicity of division and multinucleation are all important predictors of implantation potential, and the consideration of these parameters in detail does contribute to better embryo selection. Some investigators have suggested that a single evaluation on day 2 or day 3 may be sufficient for morphological selection of embryos [20]. However, if transfer is delayed until day 5 or 6 of development, the value of detailed cleavage stage morphology assessment is questionable. It is after all easier to judge with absolute certainty if an embryo *is* a blastocyst on day 5 than it is to guess on earlier days whether the embryo *is likely to become* a blastocyst. This idea is graphically presented in Figure 30.7. At the same time, reaching the blastocyst stage in vitro does not guarantee viability and further development in vivo, nor does it show chromosomal integrity. Moreover, delaying transfer is not without risk (Kälén, *et al.*, 2010). This is where the need for better means of embryo quality assessment becomes obvious and where 'OMICS' and morphokinetics may come to play a significant role in the future.

Table 30.2 Biological significance and clinical correlations of different morphological abnormalities in human eggs and embryos following ovarian hyperstimulation and in vitro culture

Development Day	Stage	Morphology	Biological Significance	Clinical Correlation
0	Oocyte	GV; 0PB	Nuclear Immaturity	Poor Prognosis Following IVM
0	Oocyte	no GV; 0PB	Nuclear Immaturity	Poor Prognosis Following IVM
0	Oocyte	Multiple cytoplasmic inclusions	Degenerative Changes	Poor Prognosis
0	Oocyte	Large PVS	Maturation failure? ZP gene deficiency?	Poor Prognosis
1	1-Cell	0PN; 0PB	Failed Fertilization	No Development Potential
1	1-Cell	1PN; 1PB (ICSI)	Parthenogenetic Activation	No Development Potential
1	1-Cell	1PN; 1PB (IVF)	Activation or fusion of pronuclei	Continue Culture & Monitor Development
1	1-Cell	3PN; 1PB	Digynic or dispermic	Polyploid
1	1-Cell	≥3PN; 2PB	Polyspermic	Mosaic
1	1-Cell	Separate Pronuclei	Cytoplasmic anomaly?	Poor Prognosis; Aneuploid
1	1-Cell	Uneven Pronuclei	Cytoplamic anomaly?	Poor Prognosis; Aneuploid
1	1-Cell	Persistent Small, Scattered Nucleoli	Cytoplasmic Anomaly?	Poor Prognosis; Aneuploid
2	2,4-Cell	Multi-nucleation	Failure of Cytokinesis; Abnormal Karyokinesis	Reduced IR
2	2,4-Cell	Fragmentation >15%; Type IV Fragments	Cytoskeletal Disorder	Reduced IR
2	2,4-Cell	Fragmentation >35%	Extensive Mosaicism	Early Pregnancy Loss
2	3-Cell	Asynchronous Division	Aneuploidy	Reduced IR
2	>6-Cell	Fast Cleavage Rate	Aneuploidy	Reduced IR
3	<6-cell	Slow Cleavage Rate	Aneuploidy	Reduced IR
3	8-16-cell	Multi-nucleation	Mosaicism	Reduced IR
3	8-16-cell	Fragmentation >15%; Type IV Fragments	Cytoskeletal Disorder	Reduced IR
3	8-16-cell	Fragmentation >35%	Extensive Mosaicism	Early Pregnancy Loss
3	7,9,10-cell	Asynchronous division	Aneuploidy	Reduced IR
3	>10-cell	Fast cleavage rate	Aneuploidy	Reduced IR

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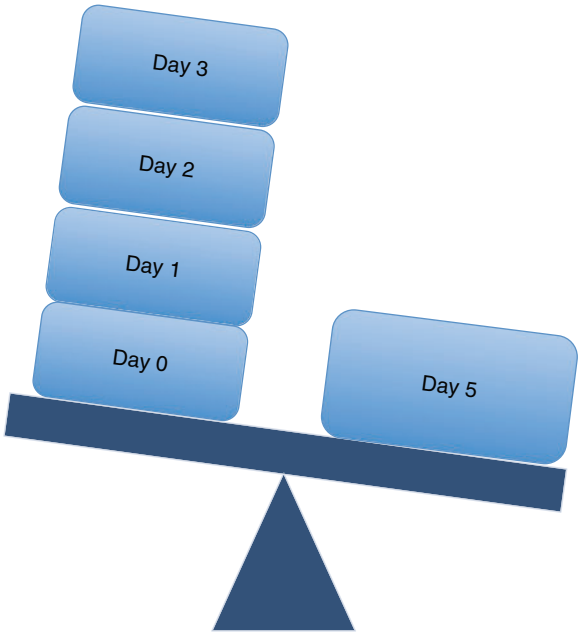

Figure 30.7 The author's opinion on the relative weight given to morphology on various developmental days in culture. For the purpose of embryo selection, blastocyst morphology provides more information than all the various morphological characteristics exhibited by eggs and embryos during the first 4 days in culture.

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Chapter 31

Cryopreservation in assisted reproduction

Jo Craig and Karen Turner

Since the early successes of IVF, constant efforts have been made to increase the efficiency of treatment cycles and the range of services provided. The use of cryopreservation techniques has been particularly effective, enabling sperm, embryos and oocytes to be stored at very low temperatures while maintaining viability for months or years [1]. The first reports of successful freezing and thawing of human embryos were made in 1983 [2], followed by pregnancies from cryopreserved blastocysts in 1985 [3]. In this chapter we will examine some of the techniques used and the different strategies that may be employed within the ART laboratory, together with some of the safety issues associated with cryopreservation. Protocols have been given to illustrate the different techniques; however, it should be remembered that these are regularly reviewed, and as new research is published they may be changed accordingly.

Theory of cryopreservation

Cryopreserved cells are stored in liquid nitrogen at −196°C. At this temperature no physiological processes take place, meaning that cells can be stored almost indefinitely. However, the freezing processes themselves can cause stress, compromising survival. The main risk is mechanical damage from ice crystal formation, and the larger the cell the more likely it is that ice crystals form in the cytoplasm. Dehydration of the cells can help prevent damage during the cryopreservation process. In order to achieve this, two complementary factors need to be considered: these are the chemical properties of cryoprotectants and the physical properties involved in the rate of cooling. Injury to the cell can also be caused by osmotic stress and 'cold shock' injury. Cold shock injury may result in membrane permeability and cytoskeletal structural changes.

Cryoprotectants

Cryoprotectants are important for the prevention of ice crystal formation and can be separated into cryoprotectants that penetrate through the cell membranes and those that do not. Most commercial cryopreservation kits consist of a basic cell culture media with the addition of a combination of penetrating and non-penetrating cryoprotectants at varying concentrations.

Penetrating or 'permeable' cryoprotectants are completely miscible with water and because they can pass through the cell membrane they have the effect of displacing water from the cell. They also help to stabilize the cell membranes and protect the cytoskeleton. At high concentrations many of the commonly used penetrating cryoprotectants, such as DMSO (dimethyl sulfoxide), are cytotoxic. Non-penetrating cryoprotectants tend to be high molecular weight sugars such as sucrose and trehalose. As they are unable to pass through the cell membrane, they act by increasing the osmolarity of the medium outside the cell and aid dehydration in that way. They are also important during thawing when they prevent damage which would otherwise be caused by excessive swelling, due to water re-entering the cells faster than permeating cryoprotectants can diffuse out.

Techniques of cryopreservation

Currently there are two commonly used procedures for cryopreservation in ART laboratories: 'slow' (computer controlled) freezing, and vitrification. Both methods have their advantages and up until recently most routine freezing was of cleavage stage embryos using slow freezing. However, advances made in the success rates of vitrification have led to increased usage, especially in the cryopreservation of blastocysts and oocytes.

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

Slow freezing

Using this method the concentration of cryoprotectant required in the medium is relatively low. During cooling this concentration increases as the water freezes, leading to further dehydration of the cell by osmosis. Optimum cooling rates will depend on the type of cell being cryopreserved. If the freezing rate is too fast then not enough water will be lost, but if it is too slow then the cells will be exposed to adverse conditions for longer than is necessary. The main factors that determine the cooling rate include the surface area to volume ratio and membrane permeability, which will change at different temperatures.

The cryoprotectants in the medium also have the effect of lowering the temperature at which the water freezes, known as 'supercooling'. This means that the formation of ice crystals in the media, known as 'ice nucleation', will not occur spontaneously until the temperature drops to below −10°C. Ice nucleation can therefore be initiated in a controlled manner away from the cells at between −5 and −10°C. This process is commonly known as 'seeding'. It is achieved by touching the straw containing the media and oocyte or embryo with either a cotton bud, or metal tweezers, dipped in liquid nitrogen.

Thawing is normally a simple procedure, with the cells being warmed rapidly from −196°C to room temperature. Cryoprotectants can then be removed by stepwise dilution, with sucrose (or other non-permeable cryoprotectant) acting as an osmotic buffer to reduce osmotic shock and increase the outflow of permeable cryoprotectants.

The main disadvantage of slow freezing is that it requires a computer-controlled cryo-machine, which is expensive to buy and has the potential to break down. Also, the standard embryo freezing program takes nearly 2 hours to run. On the positive side, the methods involved are simple to learn and give easily reproducible results, with proven success rates.

Cryo-machine

The specific cryo-machine used in our clinic in Oxford is the Planer Cryo MRV, which cools embryos by pumping liquid nitrogen vapour from a reservoir tank into the freeze chamber at a controlled rate. Other systems such as the CryoLogic Freezer work by placing a cryo-chamber into a bath of liquid nitrogen and then using a heating element to adjust the cooling rate of the embryos. Both methods are equally effective as long as the machines have been validated to ensure the temperature curves are being followed correctly and they are serviced at the recommended intervals.

Freezing protocol for cleavage stage embryos (used at the Oxford Fertility Unit)

Solutions (Sydney IVF Cryopreservation Kit, Cook Medical, Australia):

F1 – Cryo Buffer
F2 – Cryo Buffer + 1.5M Propandiol
F3 – Cryo Buffer + 1.5M Propandiol + 0.1M Sucrose

Method

Embryo manipulations take place at room temperature, with the embryos first being loaded into F1. They are then moved sequentially at 30s intervals into F2. When the first embryo has been in F2 for 10 minutes, it is moved into F3 and immediately loaded into a labelled straw (CBS™ Embryo Straw, CryoBioSystem, Paris, France). First a small amount of media is aspirated into the straw, followed by a small air bubble and then the media containing the embryo. Another air bubble is then taken up and then more media until the straw is filled completely. The straw is sealed with the labelled plug, as shown in Figure 31.1, and the process repeated with the remaining embryos. The straws containing the embryos are then loaded into the cryo-machine and the freeze program started.

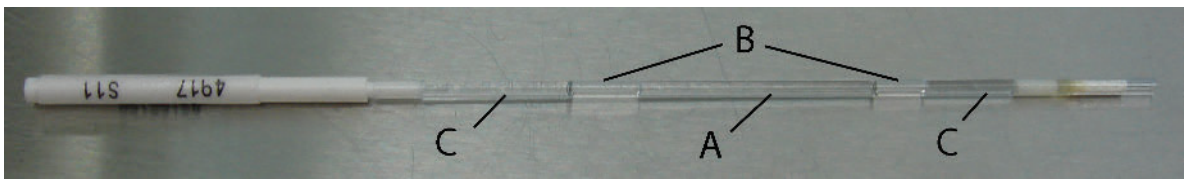

Figure 31.1 Loaded embryo freeze straw (A = media plus embryo, B = air bubbles, C = media). Ensuring there is media at both ends of the straw prevents liquid nitrogen from getting into the air spaces which could cause the straws to explode upon thawing.

Freezing program

Start temperature: +16°C, hold until embryos are loaded
Ramp 1: cool at −2°C per minute to −7°C
Ramp 2: hold at −7°C for 5 minutes before and after seeding
Ramp 3: cool at −0.3°C per minute to −30°C
Ramp 4: cool at −50°C per minute to −150°C
Ramp 5: hold at −150°C for removal of embryos

When the freeze program has been completed, the straws are removed and immediately plunged into liquid nitrogen before being transferred to the main storage vessels.

Thawing protocol for cleavage stage embryos (used at the Oxford Fertility Unit)

Solutions (Sydney IVF Thawing Kit, Cook Medical, Australia):

T1 – Cryo Buffer + 1.0M Propandiol + 0.2M Sucrose
T2 – Cryo Buffer + 0.5M Propandiol + 0.2M Sucrose
T3 – Cryo Buffer + 0.2M Sucrose
T4 – Cryo Buffer

Method

The straw containing the embryo is checked for the correct patient details and removed from liquid nitrogen. It is held in air at room temperature for 30 seconds before being plunged into a waterbath at 30°C for 40 seconds. The sealed ends of the straw are cut off and the embryo decanted into a sterile dish. Once the embryo has been located, it is removed to T1 for 5 minutes, then T2 for 5 minutes. This is followed by 10 minutes in each of T3 and T4. These manipulations take place at room temperature and then the embryo is assessed for survival (Fig. 31.2) and returned to normal culture conditions.

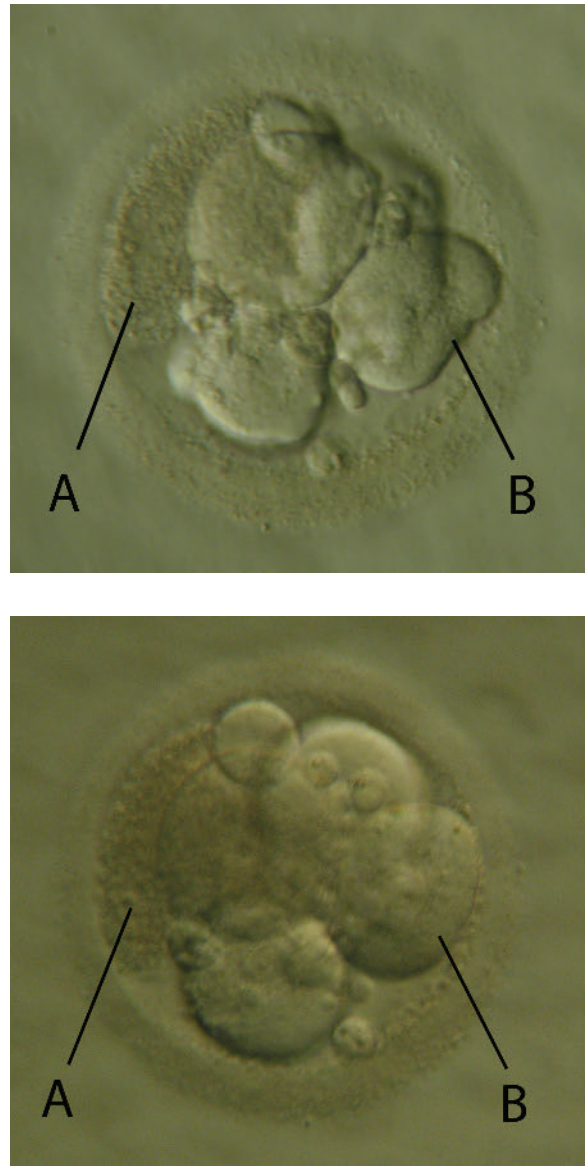

Figure 31.2 Embryo during and after thawing (A = lysed cell, B = cell showing shrinkage and re-expansion).

Vitrification

With vitrification, instead of the water becoming crystalline during the cooling process, it becomes vitreous – that is an extremely viscous liquid with the properties of a solid. The rapidity of the cooling process and the high concentration of cryoprotectants mean that the water molecules are unable to form themselves into the lattice structure of ice crystals, thus avoiding damage to the cells. The cell is placed in a hyper-osmotic medium which results in very fast dehydration, and is then cooled rapidly by placing it onto a metal block in liquid nitrogen. In order to obtain the ultra-rapid cooling rates required, very small volumes are used, typically around 1µl. The whole procedure takes only a few minutes and no mechanized equipment is involved, making it an attractive option for the laboratory. However, meticulous timing is required due to the high toxicity of the cryoprotectants at the concentrations used, which together with the very small volumes involved, means that this method requires higher skill levels than 'slow' freezing and results can be more operator dependent.

A number of different devices have been tested in order to facilitate the small volumes required for the

rapid cooling rates, such as electron microscope grids, and specially manufactured carriers such as *Cryoloops* and *Cryotips*. The method currently employed in Oxford uses CVM FibrePlugs™ (CryoLogic, Victoria, Australia), which have the advantage of being relatively easy to handle and the vitrification is achieved via a cooled metal block rather than direct contact with liquid nitrogen.

The use of multiple cryoprotectants allows a lower concentration of each to be used, thus minimizing cytotoxic effects. This can be combined with a two-step protocol to enable cells to equilibrate in a lower concentration of cryoprotectant before exposing them to the higher concentration for a very short time. Warming of the cells must also take place quickly, otherwise ice crystals may form and cause damage. Due to the small volumes used, the samples are very sensitive to temperature fluctuations. Also, it is important that cryoprotectants are rapidly diluted and removed to avoid their cytotoxic effects.

Improved success rates have been reported for the vitrification of blastocysts and oocytes compared to slow freezing. The advantage for embryo cryopreservation is less well proven, complicated by the variation in success rates reported with cleavage stage embryos. However, as more and more laboratories are culturing to the blastocyst stage before freezing, with vitrification the method of choice, it seems unlikely that it will be considered viable to also maintain programmable cryo-machines. It should also be noted that embryos which have been biopsied for the purpose of preimplantation genetic diagnosis (PGD) or chromosome screening (PGS) appear to have a better survival rate with vitrification.

Vitrification protocol for blastocysts (used at the Oxford Fertility Unit)

Agitation of the blastocyst during the equilibration stage causes the blastocoel to collapse, which has been shown to improve the success of vitrification [4].

Solutions (Sydney IVF Blastocyst Vitrification Kit, Cook Medical, Australia):

V1 – Cryo Buffer
V2 – Cryo Buffer + 8% DMSO (v/v) + 8% ethylene glycol (v/v)
V3 – Cryo Buffer + 16% DMSO (v/v) + 16% ethylene glycol + 0.68M trehalose

Method

The procedure is carried out on a heated stage at 37°C. The cryobath is filled with liquid nitrogen and the metal block allowed to cool. The blastocyst is washed in the first well of V1 and then transferred to the second well of V1. It is then moved to V2 for 2 minutes, while being agitated to make it collapse. Finally the blastocyst is moved to V3, immediately loaded onto the fibreplug (Fig. 31.3) and quickly touched on the surface of the metal block. It is held here for 10 seconds and then inserted into the chilled outer sleeve (Fig. 31.4).

Warming protocol for blastocysts (used at the Oxford Fertility Unit)

Solutions (Sydney IVF Blastocyst Warming Kit, Cook Medical, Australia):

W1 – Cryo Buffer + 0.33M trehalose
W2 – Cryo Buffer + 0.2M trehalose
W3 – Cryo Buffer

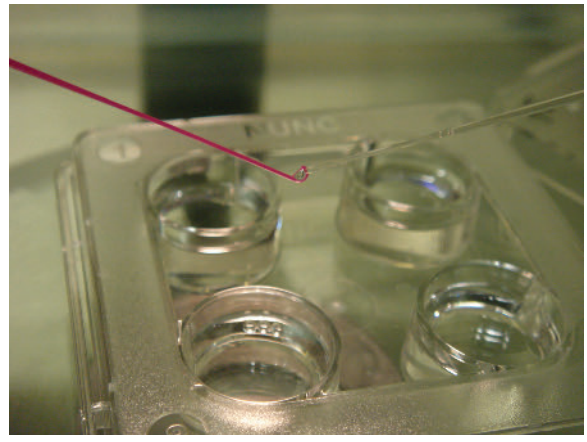

Figure 31.3 Vitrification loading.

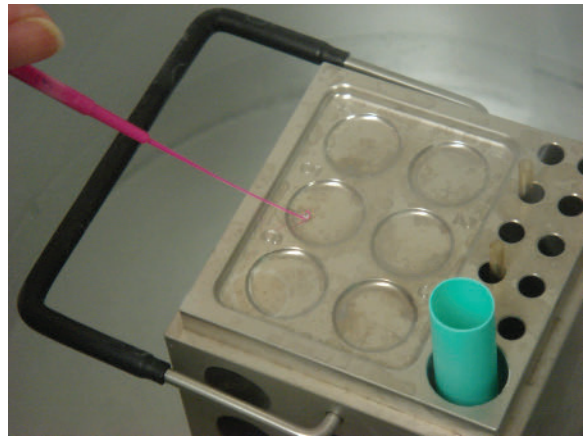

Figure 31.4 Vitrification cold block.

Method

Solutions are warmed to 37°C and the procedure carried out on a heated stage. The fibreplug is loosened from the outer sleeve while still in liquid nitrogen and then placed as quickly as possible into W1. The bead of medium will dissolve and the blastocyst sinks to the bottom of the well. The blastocyst is moved immediately to the second well of W1 for 5 minutes and washed in the solution. At this stage it is normal for the blastocyst to be fully collapsed. It is then transferred to W2 and W3 for 5 minutes each before being returned to normal culture conditions, where it should start to re-expand within 1 to 2 hours. The appearance of viable and non-viable blastocysts can be seen in Figures 31.5–31.8.

Cryopreservation of embryos

Embryos may be frozen either at the zygote stage (while the pronuclei are still visible), at the cleavage stage or at the blastocyst stage. Embryos are most often frozen as being surplus to a current IVF treatment cycle and are important in maximizing the pregnancy potential of each oocyte stimulation and recovery cycle. They may also be frozen if the patient is unable to have a fresh embryo transfer, for example due to the risk of severe ovarian hyperstimulation syndrome. Embryos may also be cryopreserved if they have been biopsied for PGD or PGS and are awaiting the results, although most genetic diagnostics are rapid enough to allow a fresh transfer.

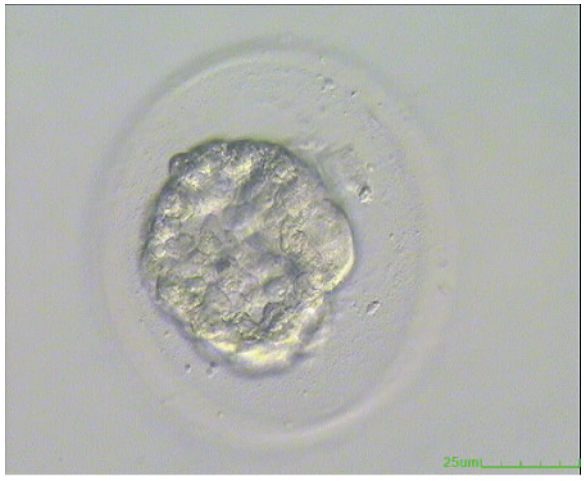

Figure 31.5 Viable blastocyst immediately after warming.

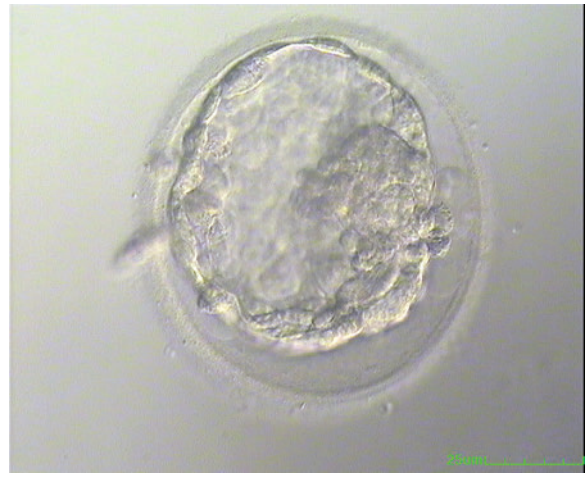

Figure 31.6 Viable blastocyst 60 minutes after warming.

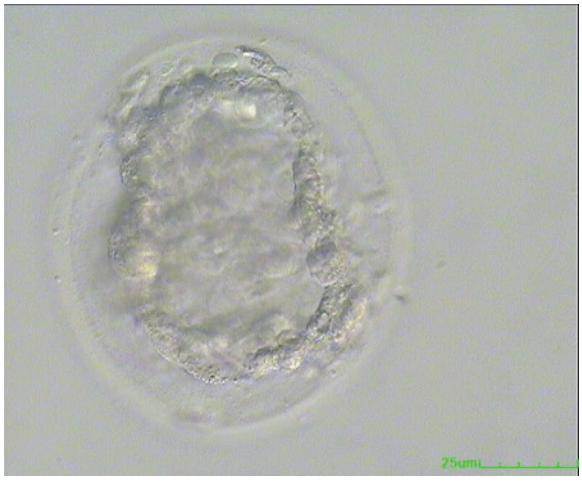

Figure 31.7 Non-viable blastocyst immediately after warming.

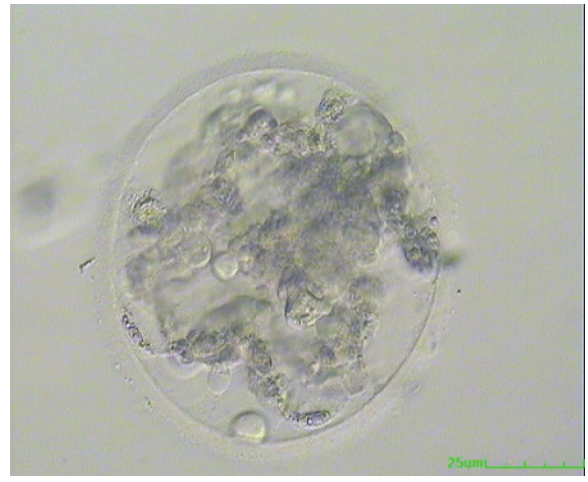

Figure 31.8 Non-viable blastocyst 60 minutes after warming.

Embryos can be created and frozen prior to treatment for malignancy that may affect the woman's future fertility, if she is in a stable relationship. However, this can prove problematic if the couple then separate, as she will be unable to use the embryos if the man withdraws his consent [5]. In the past the only option for a single woman in this situation would be to have her oocytes fertilized with donor sperm and then freeze the resulting embryos, but again this could cause complications in future relationships. The obvious answer for these women is to freeze their oocytes, but until recently this was not considered a viable option as the success of oocyte freezing was not as well established as embryo freezing [6].

The stage at which the embryos are frozen will depend on the reason for freezing and the policy of the clinic. If embryos are cryopreserved at the 2PN stage, then they can all be frozen as long as this is done during the time while the pronuclei are still visible and the embryos are chromosomally stable. Freezing at this stage can give a survival rate of around 90% [7], but the quality of the embryo remains unknown – or even whether it is capable of undergoing cleavage – and consequently a significant number of non-viable embryos may be stored. Cryopreservation at the 2PN stage is most suitable for cases where embryos are being created for storage prior to treatment for malignancy in order to conserve as many embryos as possible.

If the embryos are frozen at the cleavage stage, then it is possible to be more selective about which embryos to freeze, thus improving the success rates of the frozen embryo replacement cycles. Survival rates are normally around 80% and embryos are commonly deemed to have 'survived' if at least 50% of the cells are intact. However, both survival and implantation rates vary widely in the literature, and therefore presumably from unit to unit. Embryos that survive completely intact will give a higher chance of pregnancy than those that only partially survive [8]. There is also a degree of flexibility when it comes to thawing cleavage stage embryos. The patient may opt only to thaw those embryos wanted for transfer and then the transfer is performed a few hours after thawing. Alternatively, depending on the number of embryos available, the pregnancy rate may be further improved by thawing more embryos than required for transfer and culturing them either overnight or to the blastocyst stage. This may be particularly suitable for patients who had a large number of embryos frozen and wish to maximize their pregnancy rate from each cycle.

The increased pregnancy rate shown with fresh blastocyst transfers [9] means that many laboratories are choosing to culture to day 5 before embryo transfer. The higher implantation rate associated with blastocyst transfers, compared to cleavage stage, means that elective single embryo transfer is often more acceptable to the patient, leading to lower multiple pregnancy rates. It is widely reported that the best survival and implantation rates for cryopreservation of blastocysts are found after vitrification [10]. Even if the patient has had fresh embryo transfer at the cleavage stage, it may be worthwhile culturing any spare embryos to the blastocyst stage before assessing for cryopreservation. In this way it can be ensured that only developmentally competent embryos are stored.

The main disadvantage of cryopreservation at the blastocyst stage is that there are fewer spare embryos to freeze than there would have been at the cleavage stage [9]. This means that there is an increased risk of having to cancel the thaw cycle because there are no surviving blastocysts. The numbers available for the patient can be increased by also freezing those slower developing embryos which reach the blastocyst stage on day 6. Although it is accepted that these blastocysts have lower implantation rates with a fresh transfer, it is likely that this can be mitigated by the improved synchronization with the endometrium after thawing [11].

Early blastocysts tend to have better survival rates, probably due to their smaller blastocoels. This has led to procedures being used to reduce the volume of fluid within the cavity of expanded blastocysts by either agitating the blastocyst to make it collapse, removing the fluid with a needle, or by pulsing with a laser. There have also been studies advocating the use of artificial hatching after blastocyst warming. Survival rates of over 80% have been quoted after vitrification, but the definition of survival is harder to quantify for blastocysts. Various morphological factors have been shown to be predictive of implantation after warming [12], and as a general rule a blastocyst that has survived should begin to show signs of re-expansion within two hours of thawing.

Cryopreservation of oocytes

Oocyte cryopreservation is the most obvious method of preserving fertility for women prior to undergoing treatment for malignancy that may affect their future fertility. Although the first live birth from cryopreserved oocytes was achieved in 1986, the technical

difficulties associated with freezing such a large cell mean that only in recent years has it become a practical undertaking in the average ART laboratory. The use of ICSI following thawing has increased the fertilization rates and vitrification may improve survival rates. However, the success rates are still open to interpretation, with the most critical factor being the age of the woman involved [6].

Other reasons for freezing oocytes may be for couples who do not want to create excess embryos for ethical reasons, or as an emergency procedure if the male partner is unexpectedly unable to provide a sperm sample on the day of oocyte retrieval. Another more recent use is in oocyte donation programmes, where cryopreservation may avoid the need for synchronizing the donor and recipient and allow for quarantining. In these circumstances, pregnancy rates similar to using fresh oocytes have been found in some laboratories [13]. The use of oocyte freezing to preserve fertility for social reasons is more controversial, and given the current variation in success rates is not yet generally recommended.

Cryopreservation of sperm

Human sperm are relatively robust when it comes to cryopreservation, due to their small size and large surface area to volume ratio. The plasma membrane is highly permeable to water, facilitating dehydration when cryoprotectant is added. Also, because the sperm head is mostly condensed DNA and contains little cytoplasm, they are less susceptible to ice crystal formation. However, due to this overall tolerance of sperm, little is done in the typical ART laboratory to optimize the cryopreservation process and survival rates are still commonly suboptimal. It should also be noted that significantly worse survival can be expected when semen parameters are abnormal [14].

The usual procedure for cryopreservation of sperm is to add cryoprotectant to the sample and then suspend it in nitrogen vapour before plunging into liquid nitrogen. Better survival rates may be achieved using a controlled rate cryo-machine, but as discussed previously, these can be expensive to buy and maintain.

Sperm may be frozen either before or after preparation without affecting its survival [15]. This is normally a matter of convenience depending on the quality of the original sample. Ejaculated sperm is usually frozen within the seminal plasma, but in cases where the count is extremely low it may first be concentrated by standard laboratory techniques such as density gradient centrifugation. Surgically retrieved sperm and retrograde ejaculations are normally washed after processing, then resuspended in a suitable culture media and frozen using the same protocols.

The cryopreservation of sperm is a routine procedure in most ART laboratories, and may be carried out for a number of reasons. These reasons can be roughly divided into three categories;

I. Preservation of fertility

One of the most important reasons for cryopreserving sperm is for men about to undergo treatment for malignant disease that may affect their future fertility, or surgery that may result in erectile dysfunction. More controversially, sperm may also be stored prior to a vasectomy.

II. Quarantine of donor sperm

In order to ensure that donor semen is free from bacterial and viral infections, the samples are routinely quarantined for at least 180 days, as it may take this length of time for an infected donor to become sero-positive. At the end of this time the sperm donor is re-screened before the samples are released for use [16].

III. To facilitate current IVF treatment

For some patients who are about to undergo IVF treatment it may be an advantage to have a frozen sperm sample available on the day of oocyte recovery. For example, men who require surgical sperm retrieval can have the procedure performed before their partner even starts the drugs for her IVF cycle. This means that she only starts treatment once it is established that there will be sperm available to fertilize the oocytes. It is similarly advantageous to freeze samples obtained from retrograde ejaculation, as the preparation process can then be carried out at a time convenient to the laboratory. In both these cases, the sample may be split before freezing so that it can be used in several treatment cycles, avoiding the need for the man to undergo multiple procedures.

Cases where the sperm count is very low (less than 0.5 million motile sperm in the sample) may also prove beneficial to freeze, in order to ensure that sufficient sperm is available on the day. It may sometimes be necessary for the man to produce a number of samples for freezing over a period of time to increase the total number of sperm available. Other samples that may be frozen are

where the man has production problems and may be unable to produce a fresh sample on the day of oocyte recovery, or in cases of geographical separation where the male partner is unable to be present at the required time.

Sperm cryopreservation protocol (used at the Oxford Fertility Unit)

Commercially bought sperm cryopreservation media consists of a basic culture medium with glycerine (15%) as the main cryoprotectant and a small amount of sucrose (< 2%). This is slowly added to the sperm sample at a ratio of 0.7 ml of cryopreservation media to 1.0 ml of sperm sample. After mixing, it is loaded into labelled straws CBS™ High Security Sperm Straws (CryoBioSystems) which are then heat sealed at both ends. The straws are suspended in nitrogen vapour for 30 minutes before plunging into liquid nitrogen.

Sperm thawing

Sperm is thawed rapidly by removing it from liquid nitrogen and allowing it to warm to room temperature. It may then be used for direct intracervical insemination, still with the cryoprotectant. If the sample is to be used for intrauterine insemination or IVF, it should first be prepared. This may be done by slow dilution with medium followed by centrifugation and further washing, or by layering onto a density gradient. The method used for post-thaw preparation will depend on whether there was any pre-freeze preparation and also the overall quality of the sample.

Patient preparation for frozen embryo replacement cycle (FER)

The thawing of the embryos needs to be timed so that the patient's endometrium is at the correct stage for implantation. This may be done in a 'natural cycle' during the normal menstrual cycle or in an artificially controlled cycle. Protocols for these procedures will vary from unit to unit and may be modified for individual patients, but the basic principles remain the same.

A natural cycle FER is appropriate for women who have regular ovulatory cycles, and it does not require her to take any drugs either before or after the embryo transfer. She will normally be scanned around day 10 of her cycle to monitor the growth of her natural follicle and endometrium. Depending on the size of the follicle she will be advised when to start to do urinary LH tests, which can be bought from most pharmacies. When she has had an LH surge, the day of the embryo thawing and transfer can be calculated. The second day after the surge can be taken as the equivalent of oocyte collection, meaning that an embryo frozen on day 3 should be transferred on the fifth day after the LH surge. The main disadvantage of natural cycles is the unpredictability of the thawing and transfer, making planning of the workload more difficult for the laboratory and the transfer day less flexible for the patient. As many laboratories may be unwilling to carry out thaws at the weekend it may mean that the cycle will need to be cancelled if the patient surges on the 'wrong' day. Also, if there is a chance that the patient may be able to conceive naturally, the couple should be advised to use barrier contraception or abstain from intercourse during the cycle – especially if more than one embryo is to be transferred.

In an artificial or 'controlled' FER cycle the patient first has her FSH and LH production switched off by using a GNRH-agonist as in a fresh IVF cycle. She is then given increasing doses of estrogen as tablets or patches and scanned around day 12–14. The embryo transfer is planned when the endometrium has reached a satisfactory thickness, usually 8 mm or more. Progesterone pessaries are started, timed to the day 0 of embryo development, such that an embryo frozen on day 3 will require the patient to have had 3 days of progesterone before the day of transfer. The estrogen and progesterone will need to be continued until 10–12 weeks of pregnancy, when the placenta will take over the hormone production necessary to maintain the pregnancy. The advantages of a controlled cycle are that it is suitable for women who do not ovulate regularly, and the timing of embryo transfer is more flexible, allowing easier planning for both laboratory and patient. The main disadvantages are that the whole cycle takes much longer and that if the woman becomes pregnant, she has to continue medication for weeks after the transfer.

There appears to be no significant difference in success rates between the two methods of patient preparation for FER [17]. The most significant factors affecting the success of an FER programme are: patient age, embryo quality, embryo survival, stage of embryo development at transfer, cryopreservation method used and the technical skill of the embryologist carrying out the freezing and thawing.

Safety issues involved with cryopreservation

Transmission of pathogens

It is known that viruses and bacteria can survive in liquid nitrogen, therefore there is a risk that pathogens could be transmitted from one patient to another during storage. This risk can be minimized by screening patients for viruses such as HIV, Hepatitis B and Hepatitis C prior to treatment, and only storing screened patients in the same tank. As the patient could be infective but not sero-positive this only reduces risk, rather than avoiding it altogether. As semen has higher risks of infection it should be stored in separate tanks to oocytes and embryos. The type of containers used for storage of the gametes or embryos will also affect the risks of infection; 'open' systems, where the gametes or embryos come into direct contact with the liquid nitrogen, provide more of an opportunity for cross-contamination than 'closed' systems. Straws such as the CBS High Security Sperm™ straws supplied by CryoBioSystems (Paris, France) allow for sterile filling of the straws which are then heat sealed at both ends. Finally, storage in vapour phase of the liquid nitrogen avoids the risk of transmission and has proved safe for sperm. However, the increased temperature fluctuations in vapour phase make it less attractive for the storage of embryos, especially those that have been vitrified and are therefore more susceptible to temperature changes.

Traceability and witnessing

It is essential that gametes and embryos are correctly identified during the cryopreservation process and accurately labelled for future use. Robust witnessing protocols should be in place at each stage during all laboratory procedures, from the initial retrieval of gametes to the final placement in the storage tanks. The samples themselves should be easily identifiable and at the minimum should be labelled with the patient's full name, date of freeze and a unique identifier. Documentation of the samples in storage should be properly maintained such that samples can be easily located and that those reaching legal storage limits can be identified.

Operator safety

Hazards to the laboratory staff should be taken into account when handling samples stored in liquid nitrogen, with suitable training and provision of appropriate safety equipment being essential. The main risks can be summarized as follows:

- Injury from samples exploding due to liquid nitrogen in the container rapidly expanding when removed from storage.
- Freeze burns due to bodily contact with liquid nitrogen or chilled surfaces.
- Suffocation due to handling of liquid nitrogen in poorly ventilated areas

Summary

While for many years, slow freezing of cleavage stage embryos was the norm, the introduction of vitrification has led to increased success in the storage of blastocysts and oocytes. The safety of children born from cryopreserved gametes and embryos is of paramount importance, and although there is some controversy in the literature, there appears to be no increased rate of adverse neonatal outcome when compared to fresh IVF pregnancies. There are few studies comparing the obstetric and neonatal outcomes of children born from the different methods of cryopreservation, but again the results that are available are reassuring [18].

Cryopreservation has become an integral part of the modern ART laboratory, increasing the efficiency of the service provided, helping to reduce the multiple pregnancy rate and giving hope to cancer sufferers whose chance of parenthood may otherwise be destroyed. It is the responsibility of the embryologist to provide the highest possible standard of practice in order to ensure the best possible outcomes from the cryopreservation procedures.

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Chapter

32 Reproductive surgery

Enda McVeigh

As a general principle, the use of surgery to correct pathological or embryological defects in the female reproductive tract (Fig. 32.1) in order to increase fecundity is preferential to assisted conception. Natural conception following successful surgery is more cost-effective, is associated with a lower multiple pregnancy rate and is reproducible without further intervention. Conditions where surgery may be appropriate include embryological defects, tubal disease, fibroids and endometriosis. In many of these conditions, surgery may not only improve fecundity but may also alleviate other symptoms, such as pain or heavy menstrual loss. It is the intention of this chapter to provide a brief overview of the use of reproductive surgery for fertility treatment.

Embryological development of the female genital tract

Genetic sex is determined at the moment of conception by the presence or absence of the Y chromosome. This, after week 6 of fetal life, will guide the subsequent development of the fetus down one of two standard pathways –male or female. Following gonadal differentiation, the presence or absence of gonadal hormone production, and other fetal factors, guide the development of the Mullerian ducts, Wolffian ducts and external genitalia. The testes secrete androgens, leading to male external genital development and differentiation of the bilateral Wolffian ducts into the vas deferens, seminal vesicle and epididymis. The testes also secrete anti-Mullerian hormone (AMH; also known as Mullerian-inhibiting substance or MIS), which causes regression of the Mullerian ducts. Fetal ovaries do not secrete androgens or AMH. Consequently, female external genital development ensues, coincident with growth of the Mullerian ducts and spontaneous regression of the Wolffian ducts.

Mullerian anomalies

Abnormal development of the Mullerian ducts can lead to a wide range of conditions. Many are subtle variations of normal Mullerian anatomy and often remain asymptomatic or require no treatment. Others are transverse or longitudinal structures and may present in a variety of ways. An understanding of the timing and sequence of embryological development of the entire urogenital system helps in understanding such conditions (Fig. 32.2). Vaginal development begins at 9 weeks of fetal life. The uterovaginal plate forms between the caudal buds of the Mullerian ducts and dorsal wall of the urogenital sinus with the upper third of the vagina developing from the paramesonephric ducts and the remainder of the vagina originating from the urogenital sinus.

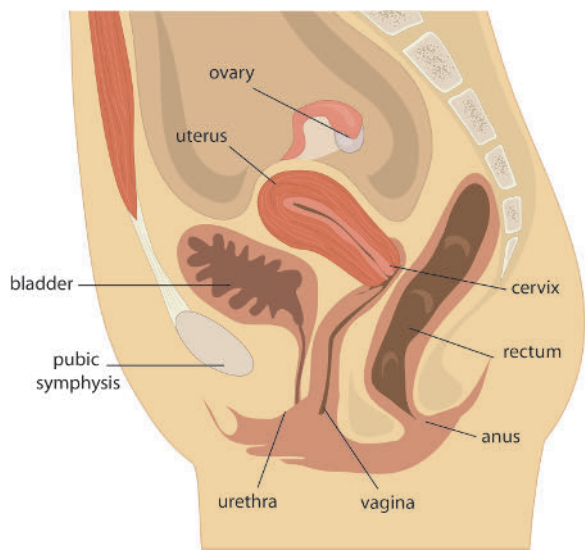


Figure 32.1 Normal pelvic anatomy.

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

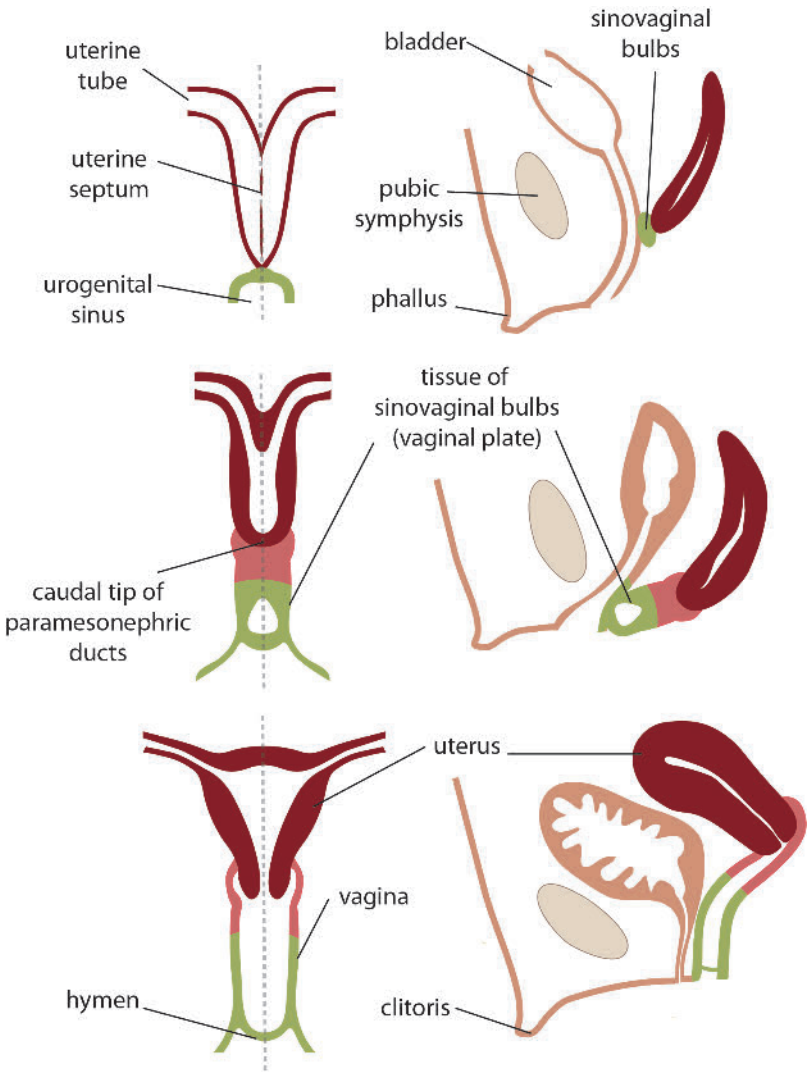


Figure 32.2 Embryological development of the female reproductive tract.

Anomalies of Mullerian system development have been classified by the American Fertility Society (AFS) (Fig. 32.3). Congenital Mullerian abnormalities generally fall into one of three groups: a normally fused single Mullerian system with agenesis of one or more parts; a unicornuate system (unilateral hypoplasia or agenesis of one Mullerian duct); or lateral fusion failures (including didelphic and bicornuate anomalies). Complete agenesis is separated in Rokitansky syndrome (also called Mayer–Rokitansky–Kuster–Hauser (MRKH) syndrome). The AFS classification describes the following distinct categories:

Class I (hypoplasia/agenesis): uterine/cervical agenesis or hypoplasia. MRKH syndrome – combined agenesis of the uterus, cervix and upper portion of the vagina.

Class II (unicornuate uterus): a unicornuate uterus is the result of complete, or almost complete, arrest of development of one Mullerian duct. Incomplete in 90% of patients.

Class III (didelphic uterus): complete non-fusion of both Mullerian ducts. The individual horns are fully developed and almost normal in size. Two cervices are present.

Class IV (bicornuate uterus): partial non-fusion of the Mullerian ducts.

Class V (septate uterus): a septate uterus results from failure of resorption of the septum between the two uterine horns. The septum can be partial or complete.

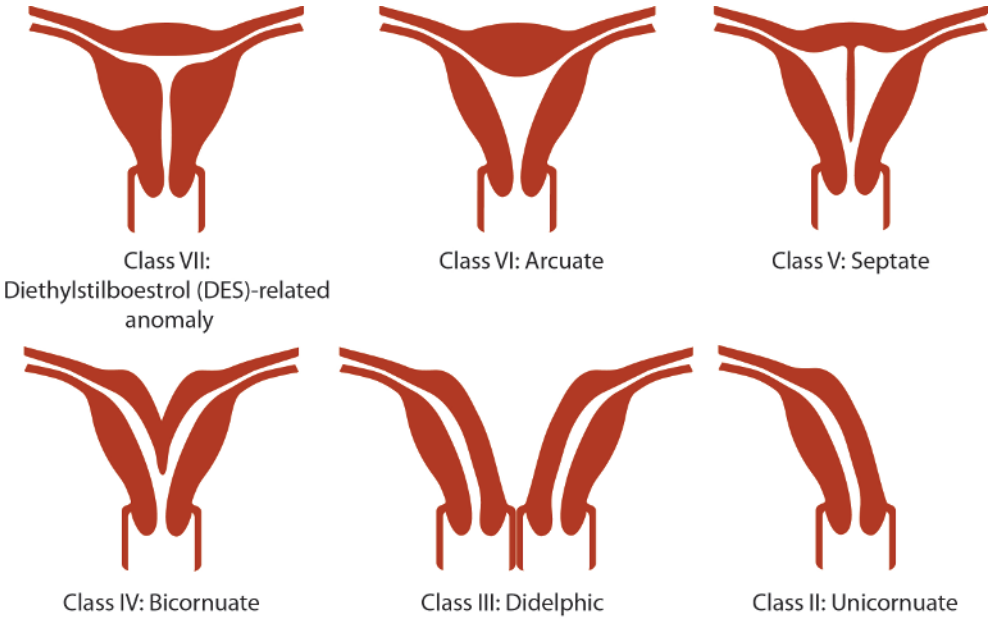


Figure 32.3 American Fertility Society (AFS) classification of Mullerian anomalies.

Class VI (arcuate uterus): an arcuate uterus has a single uterine cavity with a convex or flat uterine fundus.

Class VII (diethylstilboestrol (DES)-related anomaly): this is seen in the female offspring of as many as 15% of women exposed to DES during pregnancy. Anomalies include uterine hypoplasia, T-shaped uterine cavity, abnormal transverse ridges, stenosis of the cervix, vaginal adenosis and increased risk of vaginal clear cell carcinoma.

Surgery to correct these embryological abnormalities is only possible and appropriate in the Class V (septate uterus) condition where simple hysteroscopic removal of the septum is possible. It is extremely important, however, that the diagnosis is correct (through the use of ultrasound or MRI) and that the uterus is truly septate and not bicornuate.

Tubal abnormalities

Any damage to the fallopian tube can prevent the sperm from reaching the oocyte, or the embryo from reaching the uterine cavity, leading to infertility and tubal ectopic pregnancy. The fallopian tube is more than a simple 'tube'. First, it has cilia to assist in transport of the oocyte and embryo. Second, it facilitates capacitation of the sperm, fertilization and early development of the zygote and embryo. Therefore, the fallopian tube may maintain patency but lose the ability to promote these other functions. 'Tests' of fallopian tube 'normality' are really only testing tubal patency.

Anatomy

The fallopian tubes are seromuscular paired tubular organs that run medially from the ovaries to the cornua of the uterus. The fallopian tubes are situated towards the upper margins of the broad ligament and connect the endometrial cavity in the uterus with the peritoneal cavity towards the ovaries on each side. The tubes average 10 cm in length (range: 7–14 cm) and can be divided into four parts (proximally at the endometrial cavity to their distal portion near the ovary): (a) the intramural or interstitial portion (from the endometrial cavity, through the uterine wall, and to the uterine cornua); (b) the isthmus (the proximal third of the fallopian tubes outside the uterine wall); (c) the ampulla (the distal two-thirds of the fallopian tubes outside the uterine wall); and (d) the infundibulum, the funnel-shaped opening to the peritoneal cavity.

The fimbria are finger-like extensions from the margins of the infundibulum towards the ovaries on each side. The intraluminal diameter varies and increases from 0.1 mm in the intramural portion to 1 cm in the ampullary portion of the tubes. The

fallopian tubes receive their blood supply from the tubal branches of the uterine arteries and from small branches of the ovarian arteries. The fallopian tubes receive sensory, autonomic and vasomotor nerve fibers from the ovarian and inferior hypogastric plexi.

Pathophysiology

The main causes of tubal disease are either pelvic inflammatory disease (PID) or iatrogenic causes. PID commonly causes tubal blockage, either proximally at the site of insertion into the uterus or distally at the fimbrial end. Less commonly, a mid-tubal segment may become occluded. Blockage at two points results in a hydrosalpinx because the continued secretions of the tubal mucosa have no drainage into the peritoneal or uterine cavities. As the hydrosalpinx enlarges, the tubal muscularis thins. The secretory and ciliary properties of the endosalpinx are eventually disrupted. The probability of pregnancy following repair of hydrosalpinges with a diameter of more than 3 cm is very poor. There is now good evidence that the presence of a hydrosalpinx during an IVF treatment cycle may inhibit implantation, possibly by the fluid in the hydrosalpinx entering the uterine cavity and causing an inflammatory response in the endometrium. As a result of this evidence it is recommended that if corrective tubal surgery is not possible prior to IVF, then a salpingectomy (removal of the fallopian tube (s)) should be carried out.

Reversal of sterilization

The ability to reverse a tubal sterilization depends upon the method used. The most commonly used method in the UK is the application of Filchie clips which cause the least 'damage' to the fallopian tube and are therefore easily reversed. Electrocautery of a segment or segments of the fallopian tube occludes the lumen and causes more damage to the surrounding tissues than placement of a ring or a clip over the mid-portion of the tube, or surgical interruption of the tube. Increasing the amount of damage to the fallopian tube may increase the success of the sterilization procedure, but also reduces the chance of achieving subsequent reconstruction sucessfully. The length of a tube after a reconstructive procedure correlates with success in terms of achieving pregnancy. Patients with tubes longer than 5 cm after reconstruction have better outcomes than patients whose tubes measure 3 cm or less.

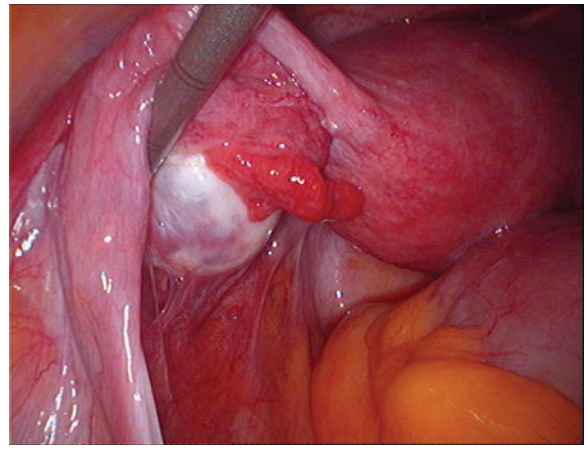


Figure 32.4 Pelvic inflammatory disease (PID). Copyright Enda McVeigh.

Pathological conditions causing tubal damage

Any inflammatory condition in the pelvis (Fig. 32.4), such as endometriosis or the sequelae of pelvic or abdominal surgery, may cause adhesions, tubal blockage or injury to the tubal mucosa and/or muscularis, resulting in tubal damage and dysfunction. In some women, cornual polyps may develop in the fallopian tube and cause a blockage that may be reversible by resection of the polyp.

Proximal tubal disease can also be caused by salpingitis isthmica nodosa. This condition is commonly diagnosed when firm nodules are found upon the fallopian tubes. Diagnosis is confirmed by histopathology. The hallmark of salpingitis isthmica nodosa is the presence of diverticula or outpouchings of the tubal epithelium, which are surrounded by hypertrophied smooth muscle. Diagnosis can only be confirmed by histology. This condition can be suspected by hysterosalpingography if proximal obstruction is present, or by a stippled appearance indicating contrast medium in the diverticular projections. It is commonly bilateral and often found in fertile women. The cause of salpingitis isthmica nodosa remains unknown. Salpingitis isthmica nodosa is found in 0.6–11% of healthy, fertile women and is almost always bilateral.

Surgery to the fallopian tube

Any surgery to the fallopian tube that is designed to restore or improve fertility should use microsurgical techniques. These techniques are more commonly used during open surgery but are increasingly carried

out by endoscopic surgery. Microsurgical technique is a delicate surgical style that emphasizes the use of magnification, fine atraumatic instrumentation, microsuturing, continuous irrigation to prevent desiccation, and pinpoint hemostasis. Specific goals are to remove pathology, restore normal anatomy and regain function with minimal damage to adjacent normal tissue. This is achieved by minimizing inflammation and preventing adhesion formation.

Intramural / interstitial obstruction

This is one of the more challenging surgeries to perform as it often involves tubal reimplantation after the resection of cornual polps. In some cases patency can be restored by hysteroscopic or radiological cannulation. The tubal ostia are visualized in the endometrial cavity with the hysteroscope or under radiological control. A small wire is inserted through the os into the intramural portion of the tube, and a small catheter is threaded over the wire. Patency can be confirmed when dye introduced through the small catheter in the intramural portion of the tube is visualized extruding through the fimbria via laparoscopy or radiologically.

Isthmic and mid-portion occlusion (including reversal of sterilization)

Isthmic occlusion can be repaired by performing an isthmic-cornual or an isthmic-isthmic anastomosis as appropriate. The damaged portion of the tube is transected perpendicular to the axis of the tube. The occluded portion of the tube is resected 2 mm at a time, initially proximally and subsequently distally, until the tubal lumen is visualized. Proximal patency is confirmed using retrograde methylene blue through a cannula into the uterine cavity. Distal patency is confirmed by threading a piece of thin suture material from the fimbrial end towards the area of anastomosis. An anchoring suture is placed in the proximal and distal mesosalpinx (isthmic-isthmic repair) or from the cornua proximally to the mesosalpinx distally (cornual-isthmic repair) to bring the two portions of the tube being reanastomosed in proximity. Four interrupted sutures are placed at the 12-, 3-, 6- and 9-o' clock positions, parallel to the axis of the tube, first within the muscularis (using a 8.0 non-absorbable suture, e.g. prolene) and subsequently onto the serosa (6.0 prolene), to bring together the proximal and distal portions of the tube. Reversal of sterilization, depending upon age, should result in pregnancy rates in the order of 80% in the first year.

Occlusion of the distal portion of the fallopian tube

This usually involves a fimbroplasty. Proximal patency of the tube should be confirmed with a preoperative hysterosalpingogram. Filling the fallopian tube with dilute dye at the time of surgery (via a cannula in the uterine cavity) facilitates identification of the entrance point in the distal, peritoneal surface of the tube that opens into the tubal lumen. The entrance point, which should be relatively avascular, is then opened using scissors, needle point diathermy or laser. The fimbria are then retracted using either sutures or thermal damage to the peritoneal surface of the tube proximal to the fimbria.

Results of surgery

Case studies indicate that 27%, 47% and 53% of women with proximal tubal blockage who had microsurgical tubocornual anastomosis achieved a live birth within 1, 2 and 3.5 years of surgery, respectively. A review of nine other case studies reported that approximately 50% of women with proximal tubal blockage who had microsurgical tubocornual anastomosis achieved a term pregnancy but did not specify the time period upon which this figure was based. Surgery is more effective in women with milder pelvic disease (stage I 67%, stage II 41%; stage III 12% and stage IV 0%).

Ectopic pregnancy

An ectopic pregnancy is one that grows outside the uterine cavity, almost always in a fallopian tube (rare sites include the ovary, cervix and broad ligament) (Fig. 32.6). As the ectopic grows, the placenta infiltrates blood vessels within the fallopian tube which can cause bleeding within the tube and bleeding into the peritoneal cavity. Further growth of the ectopic can rupture the fallopian tube causing substantial intraperitoneal blood loss. This is the commonest cause of maternal death in the first trimester, and accounts for 9% of maternal deaths in the UK.

An ectopic pregnancy may be suspected on clinical grounds, but making the diagnosis can be difficult because the presentation is so variable and can mimic that of a miscarriage. There may be a history of lower abdominal pain with a small amount of vaginal bleeding at 4–6 weeks' gestation. On vaginal examination, there may be cervical excitation and tenderness in the vaginal fornices; the cervical os

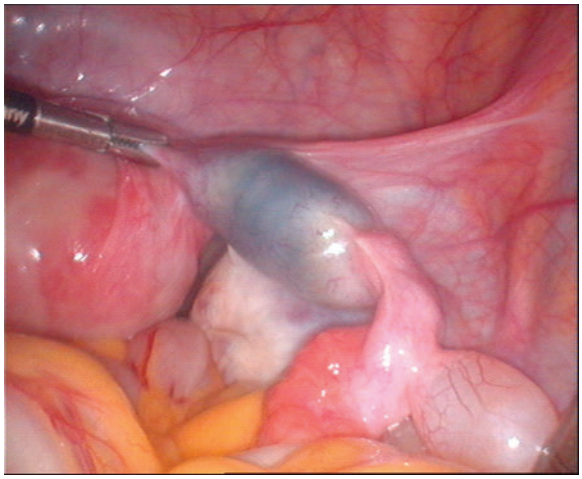


Figure 32.5 Ectopic pregnancy. Copyright Enda McVeigh.

remains closed. Alternatively, the woman may not have any symptoms or physical findings. The urinary pregnancy test is usually positive. Modern monoclonal based urine tests can detect hCG at 25 IU/l, a level reached 9 days post-conception, i.e. on day 23 of the menstrual cycle assuming ovulation occurred on day 14. Risk factors for ectopic pregnancy include previous PID, smoking, age, previous spontaneous miscarriage, previous medical termination, history of infertility or previous use of IUCD.

A transvaginal ultrasound scan should be performed if the diagnosis is suspected. The complete absence of an intrauterine gestational sac with a positive pregnancy test increases the probability of an ectopic pregnancy unless the pregnancy is not sufficiently advanced for the sac to be seen on ultrasound. Levels of hCG tend to be static or the rise is less than double over a 48-hour period if the pregnancy is ectopic. A single level above approximately 1500 IU/l in association with an empty uterus on ultrasound is highly suggestive of an ectopic pregnancy. The best diagnostic test is laparoscopy; occasionally, however, if the pregnancy is not sufficiently advanced, the ectopic pregnancy is too small to be seen in the fallopian tube. There is also a view that a laparoscopy should only be performed once a miscarriage has been excluded because of the surgical and anaesthetic risks associated with such a procedure.

Once an ectopic pregnancy is diagnosed at laparoscopy, a salpingectomy (removal of the fallopian tube) is usually then performed. In some cases, a salpingostomy (conservative tubal surgery) may be performed instead.

Uterine disorders

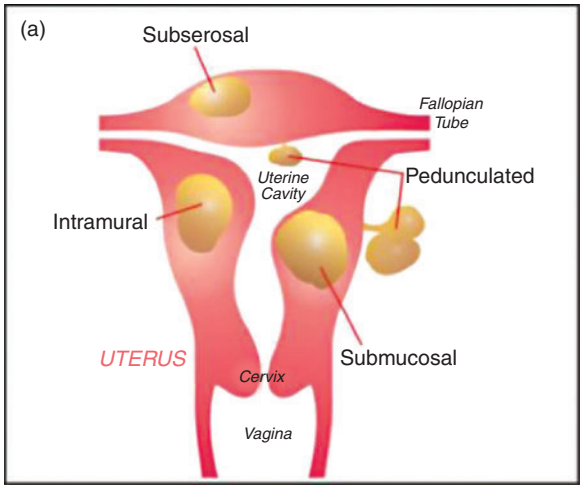
Submucous leiomyomata, congenital uterine abnormalities, endometrial polyps and intrauterine adhesions are all potential causes of infertility. The presence of a fibroid that distorts the fallopian tubes will lead to tubal infertility. Distortion of the uterine cavity, by a fibroid, a septum or a congenitally misshaped uterus can lead to implantation failure and/or recurrent miscarriage. Recent evidence has also suggested that intramural fibroids may also inhibit implantation to a certain degree.

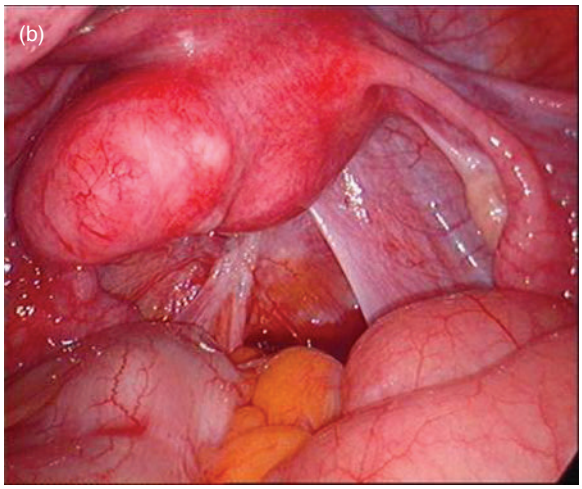
Uterine fibroids (leiomyomas)

Fibroids are benign, well-circumscribed, smooth muscle tumours of the uterus. Most women will have more than one fibroid, varying in diameter from 1–10 cm, which are typically found in the following locations (Fig. 32.6a): subserosal – may cause pressure symptoms on the bladder or bowel; if pedunculated, can be difficult to distinguish from an ovarian tumour; intramural – may similarly cause pressure symptoms; associated with infertility and heavy periods; and submucosal – associated with infertility, recurrent pregnancy loss and heavy periods; if pedunculated, may occasionally extrude through the cervical os.

The diagnosis is usually apparent on bimanual and/or abdominal examination, on the basis of finding an enlarged uterus with attached swellings. The principal differential diagnosis is an ovarian tumour. As a general rule, the uterus is felt separately on vaginal examination if an ovarian tumour is present, but not if the structures are adherent to each other. Ultrasound can usually distinguish fibroids from ovarian tumours (Fig. 32.6b and c). If not, MRI (Fig. 32.6c) is clinically useful but, sometimes, it may be necessary to perform a laparoscopy to distinguish between the two pathologies.

A GnRH agonist will usually shrink fibroids, but this class of drug has the disadvantage that treatment cannot be continued indefinitely because of the associated bone mineral density; the woman is unable to conceive while taking the GnRH agonist as it prevents ovulation, and in addition, the fibroids tend to regrow to their original size when treatment is discontinued. Uterine artery embolization (UAE) is becoming increasingly popular as an alternative to surgery. This involves blocking the blood supply to the fibroids using an angiographic technique which places particles into each uterine artery via a catheter. UAE is similar to the well-established technique of treating





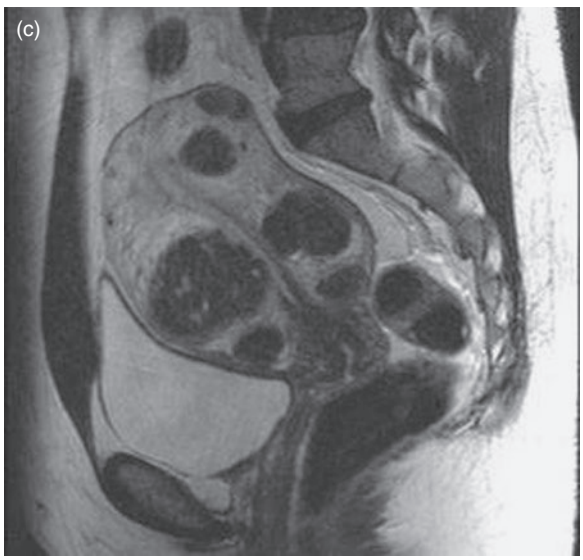


Figure 32.6 Uterine fibroids: (a) general descriptors, (b) fundal fibroids, and (c) an MRI of uterine fibroids. Copyright Enda McVeigh.

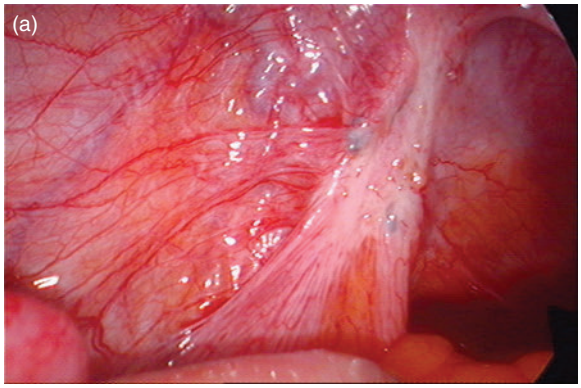
massive postpartum hemorrhage. Following embolization, the fibroids usually shrink bringing symptomatic relief, i.e. decreased menstrual bleeding and fewer pressure symptoms. Complications include arterial injury at the site of catheter insertion, severe pain due to uterine ischaemia, infection, ovarian damage and thromboembolism. A few deaths have been reported from uterine infection and pulmonary embolism. There is no consensus regarding the suitability of the technique for women who wish to conceive. Numerous pregnancies have been reported in women who have had UAE, but there still exist concerns regarding possible adverse effects on myometrial strength and ovarian function, which might affect a woman's chances of conceiving, carrying the pregnancy and delivering normally.

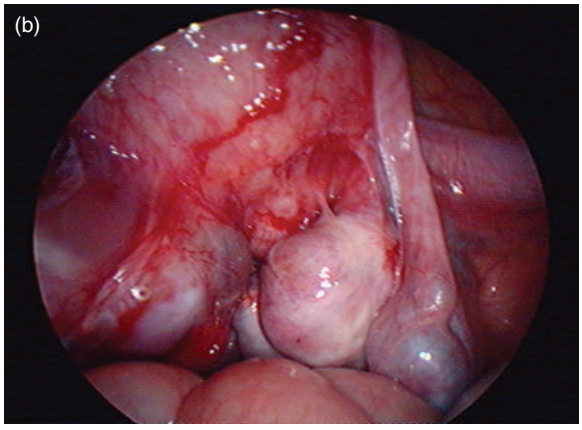
Myomectomy (performed at laparotomy or increasingly laparoscopically) involves the removal of pedunculated, subserosal and/or intramural fibroids, and closure of any defects left in the uterine wall. Surgical complications are unusual. In exceptional circumstances, however, a hysterectomy may be necessary because of uncontrollable blood loss. Fibroids in the uterine cavity should be removed hysteroscopically.

Endometriosis

Endometriosis (Fig. 32.7) is defined as the presence of endometrial-like tissue in extrauterine sites. This is a complex genetic trait which affects up to 10% of women during their reproductive years. The symptoms associated with the disease include severe dysmenorrhoea, chronic pelvic pain, ovulation pain, deep dyspareunia, cyclical symptoms related to the involvement of other organs (e.g. bowel or bladder) with or without abnormal bleeding, infertility and chronic fatigue. However, the predictive value of any one symptom or set of symptoms remains uncertain, as each can have other causes (e.g. irritable bowel syndrome) and a significant proportion of affected women are asymptomatic.

Endometriosis and infertility are associated clinically. Medical and surgical treatments have different effects upon a woman's chances of conception, either spontaneously or via assisted reproductive technologies. Medical treatments for endometriosis are contraceptive. Most studies indicate that surgery at any stage of endometriosis enhances the chances of natural conception. The most commonly affected sites are the pelvic organs and peritoneum, although distant sites





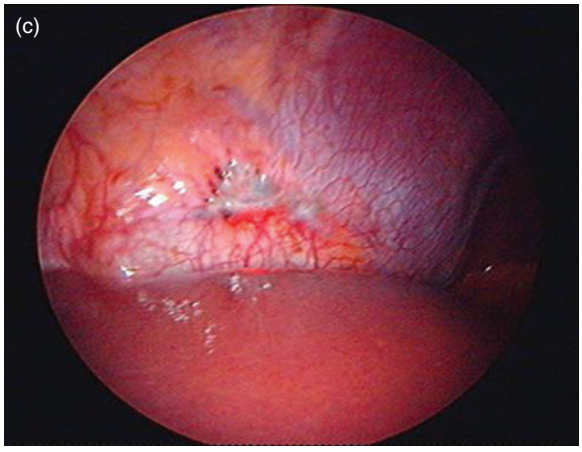


Figure 32.7 Endometriosis. (a) Endometriosis on the utero-sacral ligament, (b) bilateral ovarian endometriosis with pelvic adhesions, and (c) endometriosis on diaphragm. Copyright Enda McVeigh.

such as the lungs are occasionally affected. The extent of the disease varies from a few, small lesions on otherwise normal pelvic organs to large, ovarian endometriotic cysts (endometriomas). There can be extensive fibrosis in structures such as the uterosacral ligaments and adhesion formation causing marked distortion of pelvic anatomy. Disease severity can be assessed simply by describing the operative findings or quantitatively, using various classification systems, but there is little correlation between such systems and the type or severity of pain symptoms.

Endometriosis typically appears as superficial 'powder-burn' or 'gunshot' lesions on the ovaries, serosal surfaces and peritoneum – black, dark brown, or bluish puckered lesions, nodules or small cysts containing old hemorrhage surrounded by a variable extent of fibrosis. Atypical or 'subtle' lesions are also common, including red implants (petechial, vesicular, polypoid, hemorrhagic, red flame-like) and serous or clear vesicles. Other appearances include white plaques or scarring and yellow-brown peritoneal discolouration of the peritoneum. Ovarian endometriomas usually contain thick fluid, like tar. They are distinguishable from simple hemorrhagic ovarian cysts because typically they are densely adherent to the peritoneum of the ovarian fossa. The surrounding fibrosis may involve the bowel. Deeply infiltrating endometriotic nodules represent another disease type extending more than 5 mm beneath the peritoneum, which may grow into the uterosacral ligaments, vagina, bowel, bladder or ureters. When such lesions grow into the vagina, they may be visible on speculum examination as 'blue-domed' cystic lesions in the posterior fornix.

The diagnosis of endometriosis is usually made on visual inspection of the pelvis at laparoscopy. Non-invasive diagnostic tools, such as ultrasound scanning, can reliably detect only severe forms of the disease, such as endometriomas. Treatment options are limited because the cause is uncertain. However, treatments may include hormonal drugs to suppress ovarian function, surgical ablation of endometriotic lesions with, if necessary and usually in cases where there is associated adenomysis (endometrial glands in the myometrium), total abdominal hysterectomy and bilateral salpingo-oophorectomy.

Finding pelvic tenderness, a fixed retroverted uterus, tender utero-sacral ligaments or enlarged ovaries on examination is suggestive of endometriosis. The diagnosis is more certain if deeply infiltrating nodules are found on the utero-sacral ligaments or in the pouch of Douglas, and/or visible lesions are seen in the vagina or on the cervix. The findings may, however, be normal.

For a woman who has completed her family, hysterectomy plus bilateral salpingo-oophorectomy and removal of all the endometriosis present offers a good chance of cure. However, surgical treatment in a woman who wishes to conceive in the future aims to be as conservative as possible, ensuring in particular that ovarian function is preserved. The aim is to remove all the endometriotic tissue and restore anatomy to normal by lysing adhesions. The standard (preferably laparoscopic) methods used are ovarian cystectomy, and tissue excision or ablation with electrodiathermy, thermal coagulation or laser. The surgical risks include those for any laparoscopic procedure, as well as damage to the ureters and bowel. Risks are increased if deeply infiltrating disease is present, particularly if there is involvement of the bowel wall.

Miscarriage

A miscarriage is defined as the spontaneous loss of an intrauterine pregnancy at less than 24 weeks' gestation. A miscarriage usually starts with painless vaginal bleeding, at which point it is defined as a threatened miscarriage. The bleeding may then become heavier with associated uterine cramps, at which point it becomes an inevitable miscarriage. Blood clots and products of conception (i.e. fetal and placental tissue) are then passed through the cervical os until the uterus is emptied (defined as a complete miscarriage). Sometimes, not all the products of conception are passed spontaneously (defined as an incomplete miscarriage) and the woman may require an evacuation of retained products of conception (ERPC) to remove what is left of the pregnancy. The operation involves passing a plastic, suction curette through the cervix into the uterine cavity. Serious operative complications, which are fortunately rare, include uterine perforation which can occur with or without intra-abdominal trauma (e.g. bowel damage), cervical tears and hemorrhage.

Excessive uterine curettage after a miscarriage, especially in the presence of infection, can lead to distortion of the strata basalis endometrium. Intrauterine scarification and synechiae develop as a result, a condition known as Asherman's syndrome. As the basal layer is damaged, this condition is extremely difficult to treat and results in a significant reduction in fertility.

Acknowledgement

The author would like to express his gratitude to Mr Hamnah Bhatti (University of Oxford) for his assistance in preparing Figures 32.1 to 32.3.

Further reading

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Chapter

33

Preimplantation genetic diagnosis

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Introduction

Preimplantation genetic diagnosis (PGD) can be considered an alternative to prenatal diagnosis for couples at high risk of transmitting an inherited disorder to their children. Essentially, oocytes or embryos, generated using in vitro fertilization (IVF) techniques, are tested for specific genetic abnormalities. Those found to be healthy are prioritized for transfer to the uterus, while those diagnosed affected are excluded from transfer. Using this strategy, the possibility of an affected pregnancy is eliminated and as a result the issue of pregnancy termination is avoided. For this reason, some patients consider PGD to be more ethically acceptable than traditional prenatal diagnostic approaches involving testing after a pregnancy has been established. Patients requesting PGD are a diverse group, some motivated by personal ethics or religious views, others by difficult reproductive histories, often with multiple affected pregnancies ending in termination. Depending on the methodologies used, PGD can be applied to the detection of single gene disorders, such as cystic fibrosis and β-thalassaemia, or to the identification of unbalanced chromosome arrangements resulting from translocations or inversions. This chapter aims to describe the current status of PGD, providing an overview of the different approaches used for diagnosis, limitations of existing technology and future prospects.

Obtaining genetic information from oocytes and embryos

In its current form, PGD is inseparable from ovarian stimulation and IVF. Ovarian stimulation is necessary in order to provide several embryos from each patient, increasing the probability that at least one genetically 'normal' embryo will be identified. In vitro fertilization provides access to oocytes and embryos prior to implantation, from which genetic material can be removed for testing. There are three stages at which genetic material may be sampled: the oocyte/zygote stage (day 0 and day 1) via polar body biopsy; the cleavage stage (day 3) employing blastomere biopsy; the blastocyst stage (day 5 and/or day 6) utilizing trophectoderm biopsy. All methods involve breaching of the membrane surrounding the oocyte/embryo (zona pellucida) to provide access to the cells within. Methods for embryo biopsy are discussed in detail in Chapter 28.

PGD using polar bodies

In essence, the polar bodies (PBs) are waste products of female meiosis – small cells containing the surplus chromosomes discarded by the oocyte after each meiotic division. The first PB can be biopsied from the mature oocyte on the day of egg collection, while the second PB is extruded after fertilization. Diagnosis based upon the analysis of the first PB is particularly attractive, as this would permit testing to be carried out before conception, permissible even in countries with highly restrictive embryo protection laws, such as Germany. Unfortunately, the possibility of homologous recombination during the first meiotic division complicates the diagnosis of single gene disorders using PBs, making it essential to analyze both PBs in order to be certain of the genetic status of the oocyte (Fig. 33.1).

An advantage of PB biopsy is that samples are obtained early during preimplantation development. This allows the IVF laboratory maximum flexibility in terms of embryo transfer. Most genetic tests can be completed within 24 hours, thus permitting transfer

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

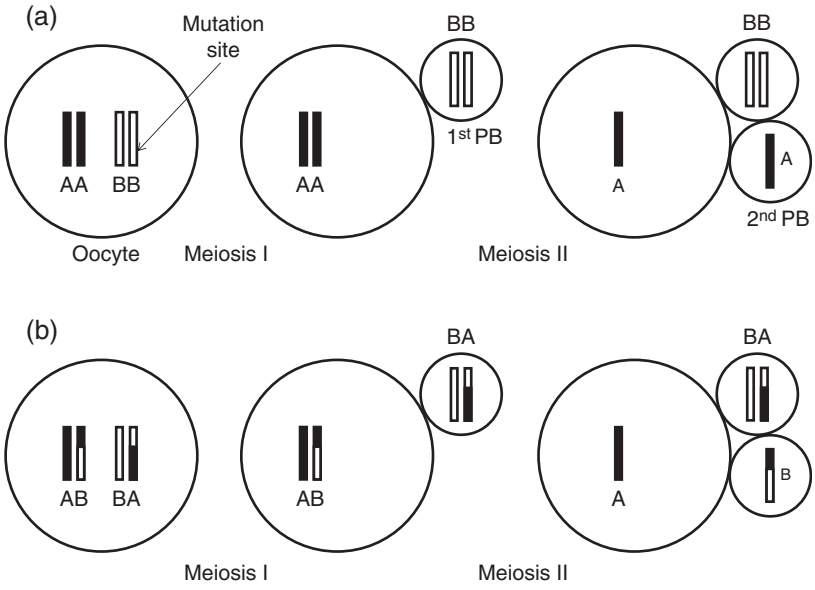

Figure 33.1 Preimplantation genetic diagnosis of a single gene disorder using polar bodies. In the case of a heterozygous woman carrying a mutation, analysis of the 1st polar body can reveal the status of the corresponding oocyte provided that recombination has not occurred (a). In this instance the detection of a mutation in the 1st polar body indicates that the oocyte is mutation free. Conversely, if the 1st polar body is found to be genetically normal then the corresponding oocyte will carry the mutation. However, if recombination occurs during meiosis I, the oocyte and 1st polar body may both be heterozygous at the mutation site (b). In this case, the genetic status of the oocyte is not resolved until the extrusion of the 2nd polar body at the end of the second meiotic division.

on day 2 or day 3 (cleavage stage) if desired. Additionally, PBs are not thought to be necessary for oocyte viability and as such they can be removed without fear of harming the oocyte. The major limitations of PGD strategies based upon PB biopsy are that they are labour-intensive (both PBs must be biopsied from all oocytes, even though some will produce embryos that fail to progress in culture) and genetic information is only obtained concerning the female gamete. No abnormalities of paternal origin can be tested.

PGD at the cleavage stage

By far the most widely utilized PGD strategy involves biopsy of one or two cells (blastomeres) three days after oocyte retrieval, at which point embryos are typically composed of 6–10 cells [8]. In theory, all cells at this stage are totipotent, able to give rise to any cell lineage including ectoderm, endoderm, mesoderm and extraembryonic tissues such as the placenta. Consequently, the removal of a cell should not have an adverse effect on the children produced. Reassuringly, follow-up studies have confirmed that there is no increase in congenital abnormality rates for children produced after PGD compared with those created using IVF [18].

The main advantage of cleavage stage biopsy over polar body analysis is that it allows analysis of both maternal and paternal genetic contributions to the embryo. Since the results of PGD are usually available within 24 hours of initiating a test, embryos can be transferred on day 4 or at the blastocyst stage. A disadvantage of analysis at this stage is the fragility of the cleavage stage embryo, which makes it essential that biopsies are performed by highly skilled embryologists. Poorly performed embryo biopsy can impair embryo development, leading to significant reductions in embryo implantation. Additionally, there is evidence that removal of two blastomeres for diagnosis, at one time a commonly used PGD strategy, reduces the potential of an embryo to develop to the blastocyst stage [3, 23]. For this reason, single cell biopsy is recommended in most cases. Another potential difficulty associated with cleavage stage biopsy is the issue of chromosomal mosaicism, a biological oddity that reduces the accuracy of chromosome analysis in embryos. The issue of mosaicism is described and discussed in detail in Chapter 34.

PGD applied to blastocyst stage embryos

An alternative to polar body and blastomere biopsy, which has been relatively little used until recently but shows great promise for the future, is blastocyst biopsy. In this case, a narrow channel is made in the zona pellucida, usually on day 3. As the embryo begins to expand, forming a blastocyst on day 5 or day 6, cells of the trophectoderm begin to herniate through the opening in the zona pellucida. These cells can be drawn into a pipette and separated from the embryo mechanically or by use of a laser. Typically 3–10 cells are obtained in this way (mean ~5 cells).

The main benefits of PGD at the blastocyst stage are that biopsy appears to have little if any impact on subsequent embryo development and that the greater number of cells obtained result in an improved accuracy for genetic tests. Together these two factors make blastocyst analysis highly attractive for the purpose of PGD. However, a drawback of this approach is that many patients do not produce sufficient numbers of high-quality embryos to be considered candidates for culture to the blastocyst stage and cannot therefore utilize this PGD strategy. Another problem is that blastocysts should ideally be transferred to the uterus on day 5, as uterine and embryonic factors are considered optimal for implantation at this point. However, with most PGD tests taking approximately 24 hours, transfer would often have to be delayed until day 6, or in the case of slow growing embryos, day 7. In order to avoid missing the optimal window for embryo implantation, blastocyst PGD cycles must employ tests of exceptional rapidity, allowing transfer within a few hours following biopsy. Alternatively, embryos may be cryopreserved after biopsy, with transfer of unaffected embryos carried out a couple of months later. The addition of cryopreservation entails an extra layer of complication and expense to PGD at the blastocyst stage and increases the risk of damage to the embryo, although this latter concern has been somewhat mitigated by the introduction of vitrification.

PGD for chromosome rearrangements

Once genetic material has been obtained from all of the oocytes or embryos produced during the IVF cycle, it can be tested in a variety of different ways depending on the specific type of diagnosis required. Tests can be broadly divided into cytogenetic (i.e. chromosomal) and molecular (i.e. single gene) diagnoses. The major indication for cytogenetic analysis is the presence of a chromosome rearrangement in one of the parents. Rearrangements, such as translocations and inversions, are problematic for gametes to process during meiosis, frequently leading to loss or gain of chromosomal segments or whole chromosomes in the sperm/oocytes produced. Embryos produced from chromosomally abnormal gametes may fail to implant, miscarry, or produce children affected by congenital abnormalities and/or mental retardation.

Chromosome rearrangements are relatively common in the general population and consequently account for a significant proportion of PGD cases. Translocations, the most frequently referred form of chromosome rearrangement, are found in approximately 0.2% of individuals. The abnormal gametes produced by patients carrying a translocation can have a negative impact on fertility, and consequently the prevalence is higher for couples seeking assisted reproductive treatment. Among infertile couples, 0.6% are found to have a translocation. For those with male factor infertility the incidence is higher still (3.1%), while 9.2% of couples with a history of recurrent pregnancy loss carry a translocation. There are two types of translocations – reciprocal and Robertsonian. Reciprocal translocations are generated when a break occurs on each of two chromosomes, followed by exchange of segments, resulting in the formation of two new derivative chromosomes. Reciprocal translocations can involve any combination of two chromosomes. Robertsonian translocations, on the other hand, are formed when two acrocentric chromosomes (13–15, 21, 22) break at a position at or very close to the centromere with subsequent fusion of their long arms. In this way, a single metacentric derivative chromosome is produced.

Until recently, the processing of biopsied cells for chromosomal analysis involved placing the material on a microscope slide and fixing the specimen using a mixture of methanol plus acetic acid or hydrochloric acid and Tween (a detergent) [13, 4]. The fixed cells were then analyzed using fluorescence *in situ* hybridization (FISH), a method involving the annealing of chromosome-specific DNA probes, labelled with different coloured fluorescent dyes. FISH probes produce a spot or 'signal' visible at the site of hybridization. The number of signals reveals the number of copies of the chromosome region tested. The reason for analyzing the chromosomes indirectly via FISH is that direct visualization of chromosomes is not straightforward in PBs and embryonic cells. Biopsied blastomeres and trophectoderm cells are almost always found to be in interphase, the chromosomes contained within a nucleus, diffuse, overlapping, and impossible to resolve as individual entities. The discrete signals produced by FISH probes are clearly visible on interphase nuclei (see Chapter 34). Although PBs are in metaphase, the morphology of their chromosomes is not compatible with conventional cytogenetic analysis (chromosomes are extremely short) and consequently FISH is needed to positively identify individual chromosomal regions in this case too. Clinical application

of FISH for PGD of chromosome rearrangements has shown significant benefits for patients, principally a reduction in miscarriage rate. In a recent retrospective review of data obtained during 192 cycles of PGD for either reciprocal or Robertsonian translocations, it was demonstrated that the miscarriage rate was reduced from 35–64% (natural conceived pregnancies) to 13% after PGD for the specific chromosome rearrangement. A healthy chromosomally normal (or balanced) conception was achieved, on average, after 1.4 cycles of PGD [7].

The most widely employed FISH strategy for carriers of reciprocal translocations involves the use of three differentially labelled probes. This is termed the 'flanking probe' strategy, as two of the three probes 'flank' the breakpoints on one of the two chromosomes involved in the structural rearrangement, while the third probe is specific for a locus on the other chromosome. The commercial availability of a wide range of locus-specific, centromeric or subtelomeric probes meant that the flanking probe approach could be used for PGD of the vast majority of reciprocal translocations.

The FISH strategy applied for the PGD of Robertsonian translocations is generally less complicated than that used for reciprocal translocations. However, centromeric probes cannot be used due to sequence homology of the satellite regions between chromosomes 13 and 21 and 14 and 22. For this reason, the PGD-FISH approach generally employed for Robertsonian translocations involves the application of two probes, each one hybridizing on one of the two chromosomes participating in the rearrangement. In cases where one of the chromosomes involved in the translocation could result in the generation of a viable trisomic pregnancy, such as chromosome 21, then two probes are ideally applied for that chromosome, ensuring maximum accuracy of diagnosis.

While FISH has been successfully applied in many PGD laboratories, it is increasingly being replaced by alternative microarray comparative genomic hybridization (array-CGH) methods. Array-CGH is described in detail in Chapter 34, but essentially it allows the copy number of all chromosomal regions to be simultaneously determined in a single test. This means that a single protocol can be used for the vast majority of chromosomal rearrangement carriers, regardless of the specific chromosome segments involved. In contrast, FISH is limited to analysis of small numbers of probes (i.e. only a few chromosomal regions can be simultaneously assessed) and as a consequence specific combinations of probes must be chosen and validated for individual patients. The preclinical workup necessary for PGD using FISH adds to the expense of the procedure and results in delays to patient treatment. As well as providing an assessment of the copy number of chromosomal regions involved in a rearrangement, array-CGH also allows evaluation of every other chromosome. Thus, any spontaneously arising aneuploidies, unrelated to the chromosome rearrangement, are also detected. This may be advantageous, particularly for patients of advanced reproductive age (i.e. late thirties or forties), who are at elevated risk of Down's syndrome and miscarriage associated with aneuploidy [1, 6].

PGD for single gene disorders

Since the completion of the sequencing of the human genome, it has become possible to design a PGD test for virtually any genetic disorder caused by the inheritance of a single defective gene. To date, more than 200 different single gene disorders have been diagnosed in human embryos. In the UK, PGD is only permitted for diseases approved for testing by the Human Fertilisation and Embryology Authority (HFEA) (see Chapter 22 for more about regulation of IVF and PGD). To date the HFEA has reviewed and approved the use of PGD for over 160 different monogenic conditions.

The first ever cases of PGD, performed two decades ago, involved cleavage stage biopsy followed by polymerase chain reaction (PCR) amplification of a specific DNA sequence situated on the Y-chromosome. This approach was used for couples at high risk of transmitting an X-linked disorder (e.g. Duchenne muscular dystrophy). Detection of amplified DNA was indicative of the presence of a Y-chromosome and therefore a potentially affected male embryo. Using this strategy, female embryos were selected for transfer and male embryos were excluded [10]. Even 20 years on, virtually all single gene PGD cases still depend on DNA amplification using PCR. However, the complexity of tests has increased significantly, reflecting a growing appreciation of the limitations of genetic diagnoses performed on single cells. In most cases, PGD for a single gene disorder now involves amplification and testing of several distinct DNA fragments in each biopsied cell. The amplification of all of these loci occurs simultaneously, a process

known as multiplex-PCR. Using this approach, the risks of misdiagnosis caused by amplification failure and contamination can be greatly reduced (as described below).

DNA contamination

One of the most serious problems facing PGD protocols based upon DNA amplification is the risk that the sample may become contaminated with DNA from a non-embryonic source. The large number of PCR cycles needed to amplify the DNA from the biopsied cell(s) to a detectable level will also amplify any contaminants present, potentially leading to errors in genotyping the embryo and misdiagnosis. In order to avoid DNA contamination derived from sperm, it is important to use intracytoplasmic sperm injection (ICSI) for all cases of single gene PGD, regardless of whether or not there is any male factor infertility. In conventional IVF cycles it is typical for multiple sperm to become bound to the zona pellucida while attempting to fertilize the oocyte. The use of ICSI eliminates the risk that such sperm will lyse during the biopsy procedure, releasing potentially contaminating DNA. For similar reasons, it is essential that all cumulus cells remaining attached to the oocyte/embryo are completely removed prior to biopsy.

As well as avoiding contamination derived from paternal (sperm) and maternal (cumulus cell) genomes, it is important to minimize the risk posed by DNA from individuals in the embryology and genetics laboratories. Gloves should be worn throughout procedures and changed regularly, particularly if they come into contact with exposed skin or items that have been touched with bare hands (e.g. door handles, light switches, etc.). Additional protective clothing, for example gowns, caps, masks and shoe coverings, are also advisable.

Biopsied cells should be washed thoroughly through several drops of a medium or buffer previously tested for DNA contaminants and then placed into microcentrifuge tubes used for PCR (certified to be DNA-free). It is usual to also collect a small amount of fluid from the final drop in which the cell was washed and to place this in a separate PCR tube. This 'negative control' is also subjected to PCR. If any DNA amplification is observed in the negative control, it indicates that contaminants had persisted through the cell washing procedure and that there is a high risk that the tube containing the cell is also contaminated.

One of the principal sources of contamination is derived, not from cells, but from the billions of DNA fragments produced during PCR amplification. Previously amplified DNA fragments (i.e. PCR products) tend to build up in the laboratory where the genetic analysis takes place and, if appropriate precautions are not taken, can find their way into future amplification reactions. This problem, known as carryover contamination, is best avoided by having a room set aside for the sole purpose of PCR setup. Ideally, pre-PCR laboratories of this type should have restricted personnel access, positive pressure filtered airflow (not shared with other rooms) and dedicated lab coats, pipettes and other equipment.

As well as introducing physical barriers against contamination, such as gloves and flow hoods, efforts can be made to remove DNA from the environment. The main methods for achieving this are chemical treatments (e.g. commercially available solutions for degrading DNA) that can be applied to surfaces and ultra-violet irradiation, which introduces chemical modifications in the DNA, preventing subsequent amplification by PCR.

A useful approach for detecting the presence of contamination is to amplify microsatellite polymorphisms from the biopsied cell(s). Microsatellites, also known as short tandem repeats (STRs), are highly polymorphic DNA sequences, having multiple alleles of varying length. If a multiplex-PCR is undertaken, amplifying a few microsatellite loci in addition to fragments encompassing the mutation sites, then it is possible to generate a simple form of DNA fingerprint for the embryo. If DNA from more than one individual is present, this is often revealed by the presence of additional STR alleles (Fig. 33.2). The advantage of this type of analysis, compared with testing of negative controls, is that it reveals the presence of contaminants within the same tube as the biopsied material, rather than a separate sample taken in parallel. Additionally, the fingerprint can sometimes indicate the source of contamination. For example, if one paternal allele and two maternal alleles are observed (instead of one allele from each parent), DNA from a cumulus cell or other maternal source may be responsible. Alternatively, if additional alleles are detected, which are not present in either of the parents, the likely source of contamination is an individual from the embryology or genetic laboratories.

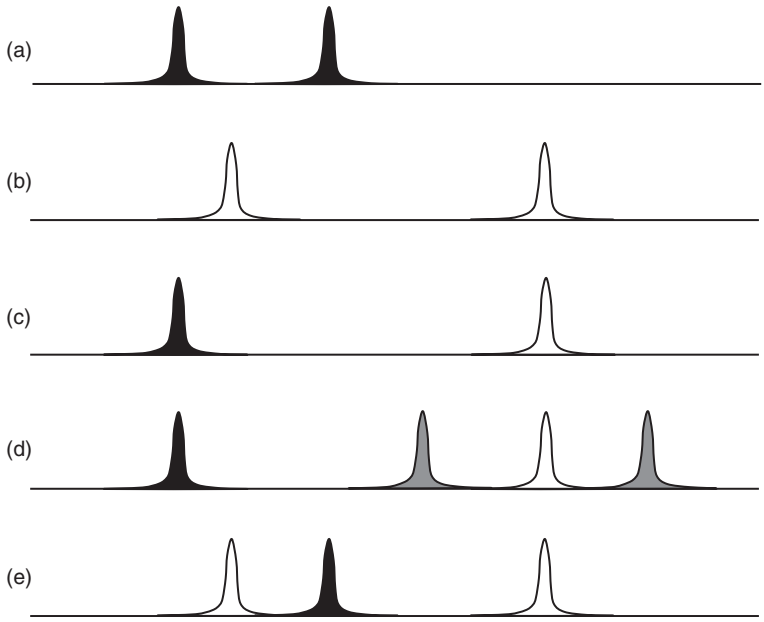

Figure 33.2 The use of hyper-variable (short tandem repeat- STR) polymorphisms for contamination detection. (a) Paternal genotype. The father is heterozygous for the STR polymorphism analyzed. (b) Maternal genotype. The mother is heterozygous for the STR polymorphism and shares no alleles in common with the father. (c) Potential embryonic genotype. The embryo has inherited one STR allele from each parent and no contaminants have been detected. (d) Result for an embryo sample with contamination. As well as alleles inherited from the parents, additional alleles from an unknown source are present, indicative of the presence of contaminating DNA. (e) An embryo sample with three parental alleles (two maternal and one paternal) instead of two. This could be explained by contamination with DNA from a maternal source (e.g. a cumulus cell) or trisomy for the chromosome carrying the mutation.

Amplification failure and allele dropout

Another challenge facing PGD for single gene disorders is the need to achieve efficient amplification of both alleles in each cell. The first misdiagnosis to affect embryo testing involved the failure to successfully amplify a Y-chromosome sequence, resulting in a male embryo affected by an X-linked disorder being incorrectly diagnosed female [12]. Since that time, diagnostic strategies have attempted to avoid situations where amplification failure could be interpreted as indicative of an unaffected embryo. Typically 5–10% of cells biopsied from embryos fail to produce any DNA amplification after PCR. This can be explained by loss of the cell during transfer to the PCR tube or the biopsy of a cell which was anucleate or contained degraded DNA.

While most of the problems facing diagnoses performed on single cells are observed to some degree in other PCR applications (e.g. contamination), one difficulty unique to amplification of DNA from single cells is a phenomenon known as allele dropout (ADO). In this case amplification failure is not total, but rather affects just one of the two alleles in the cell. Obviously, this is of great diagnostic relevance when the cell is heterozygous, as ADO produces an appearance of homozygosity. For dominant disorders, ADO of the mutant allele could potentially lead to an affected embryo being diagnosed normal. The frequency of ADO varies widely and appears to show some locus specificity, but typically 5–10% of single cell amplifications are expected to display this phenomenon. There is evidence that ADO may be caused by incomplete cell lysis, preventing access of PCR reagents to the DNA template, or by a double stranded break in the DNA of one of the alleles in the cell, preventing PCR amplification. Amplification failure and ADO are more common among cells derived from morphologically poor quality embryos, also suggestive that DNA damage may be a factor [14].

At this time, no method for eliminating ADO has been devised. However, the diagnostic impact of the problem can be alleviated by performing linkage analysis in addition to direct mutation detection. Linkage analysis involves the testing of polymorphisms located in close proximity to the mutant gene. DNA samples from the parents and other family members are analyzed for several such polymorphisms in the hope that one or more can be identified having alleles consistently associated with the disease causing mutation. Although these alleles do not actually cause the disease, they are inherited along with it and can therefore be used as markers of the disease (Fig. 33.3). Of course, DNA fragments containing linked polymorphisms are just as likely to be affected by ADO as fragments encompassing mutation sites. However, it is

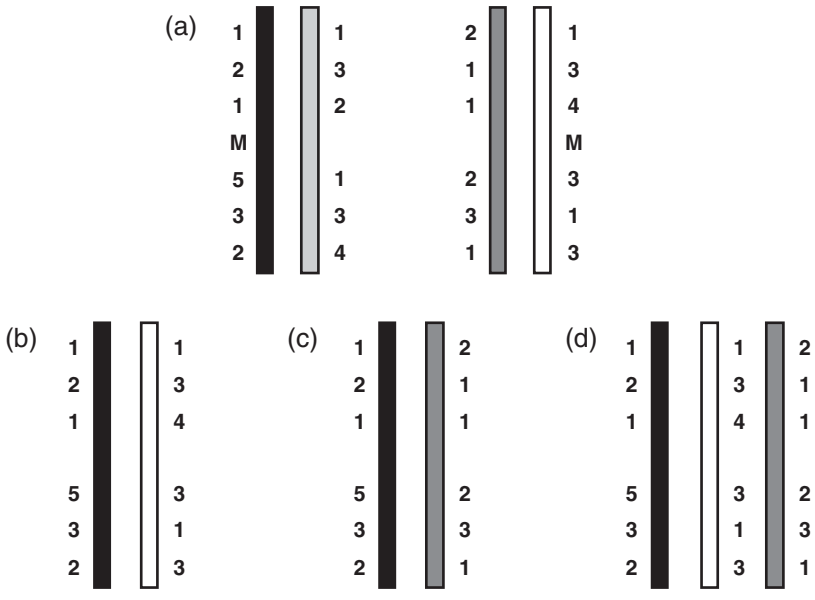

Figure 33.3 The principle of linkage analysis. Rather than direct detection of the mutation itself (labelled 'M'), the status of the embryo is inferred by analysis of polymorphisms in the vicinity of the affected gene (i.e. linked polymorphisms). Standard PGD protocols often involve analysis of several linked polymorphisms in addition to the mutation site. PGH strategies typically include evaluation of larger numbers of polymorphisms, often ten or more, and do not usually assess the mutation site. Karyomapping involves the analysis of hundreds of thousands of polymorphisms, not just in the vicinity of the mutant gene, but scattered throughout the genome on every chromosome. In this example, the inheritance of parental chromosomes (a) is inferred from the inheritance of six polymorphisms. (b) An embryo that has inherited affected parental haplotypes. (c) An embryo which has inherited one parental chromosome carrying a mutation and one unaffected chromosome. (d) An embryo that has inherited haplotypes from three distinct parental chromosomes, indicating that it is trisomic.

statistically unlikely that the linked polymorphism(s) and the mutation site will both suffer ADO affecting the same chromosome in the same amplification reaction [2, 5, 15]. Most often the polymorphisms used for linkage analysis are hyper-variable STR loci that, as described above, also provide a simple DNA fingerprint assisting in the detection of DNA contaminants.

Some PGD protocols have been designed, in which direct mutation detection has been completely abandoned in favour of analysis of multiple linked polymorphisms flanking the affected gene. This approach allows assembly of a haplotype, a group of alleles belonging to contiguous polymorphisms, spanning an area of DNA. Diagnosis relies on tracking the inheritance of the haplotype belonging to the chromosome carrying the mutation from parent to embryo (Fig. 33.3). This method, known as preimplantation genetic haplotyping (PGH), has the benefit that a single protocol can be used for all patients with the same disease [16, 17]. The ability to use the same PGD protocol for multiple patients has the potential to reduce the high costs and extended waiting times associated with the development of unique, patient-specific tests. However, the initial development of PGH strategies can actually be more time consuming and expensive than setting up a standard PGD protocol, and for that reason PGH is usually only applied to relatively common diseases, where multiple requests for PGD are anticipated. Furthermore, linkage-based strategies such as PGH ideally require DNA samples from additional relatives (e.g. siblings, parents or children of the affected couple). Analysis of these samples is necessary to confirm which alleles are inherited along with the mutation. Unfortunately, in almost one-third of PGD cases, no appropriate additional DNA samples are available, either because no family members are available for testing or because the patient has a *de novo* mutation. This problem can sometimes be overcome by testing individual sperm, but this strategy is only applicable if the male is the mutation carrier.

Extension of the indications for PGD and ethical questions

When PGD was first devised, it was anticipated that its primary role would be as an alternative to prenatal testing for patients opposed to pregnancy termination. However, in recent years the indications for PGD have been extended beyond diagnosis of the disorders

typically tested using chorionic villus sampling and amniocentesis. The first expansion of PGD indications involved application to diseases of late onset and incomplete penetrance, most notably cancer predisposition syndromes such as dominant breast cancer predisposition (BRCA1 and BRCA2) and familial colon cancer (familial adenomatous polyposis coli and hereditary non-polyposis colorectal cancer) [2, 19]. Although prenatal testing is available for many of disorders of this type, uptake has traditionally been low. Since a mutation carrier may remain healthy for many years and, in some cases, may avoid development of the disease altogether, many patients consider pregnancy termination to be too drastic an option. On the other hand, for some, selection of embryos prior to the initiation of pregnancy may be considered more acceptable and provides an opportunity to permanently eliminate the disease from the family.

A more significant departure from the original use of PGD has been the introduction of human leucocyte antigen (HLA) testing of embryos. HLA testing has been utilized in cases where a family has a child affected by a blood disorder, such as sickle cell anemia, β-thalassaemia or leukaemia [21, 22]. The only way to achieve a permanent cure for the affected child is to perform haematopoietic stem cell transplantation (e.g. bone marrow transplant or transfer of stem cells from blood taken from the umbilical cord of a newborn baby). However, in order for the transplantation to be successful, the new cells must express the same antigens on their cell surface as the cells of the affected child (i.e. they need to be HLA-matched). Finding an HLA-compatible stem cell donor in the general population is extremely challenging. However, siblings of the affected child have a relatively good chance of having an identical HLA-type (almost 1 in 4). In vitro fertilization, in which multiple sibling embryos are produced at the same time, provides a good chance of finding at least one that is HLA-matched. In the case of inherited forms of anemia, the embryos are biopsied, screened for the familial mutation(s) and also HLA-typed. The only embryos transferred to the uterus are those that are unaffected by the disorder and also HLA-matched to the sibling requiring stem cell transplantation. If a pregnancy is successfully established, then blood is collected from the discarded umbilical cord at birth and used in an attempt to cure the affected sibling. In the case of sporadic diseases (e.g. leukemia), embryos are only tested for their HLA status, no mutation testing is necessary. Unfortunately, the chances of success each cycle are relatively low due to the low chances of an individual embryo produced using IVF implanting, coupled with the fact that most of the embryos produced are not suitable for transfer due to genetic considerations. For a recessive disorder, on average, only three out of every sixteen embryos will be both unaffected and HLA matched. Nonetheless, utilization of this approach is increasing and multiple children have been cured, who otherwise would have died of their conditions.

The extension of embryo testing, into areas not traditionally covered by prenatal diagnosis, has led to ethical debate concerning the appropriateness of using PGD technology for these new indications. This book chapter is too short to permit anything other than a cursory examination of these arguments, but it is worth briefly touching on the main concerns that have been raised. In the case of PGD for diseases of late onset, some have argued that elimination of affected embryos is unacceptable, since the individuals they have the potential to produce might lead full, productive and healthy lives for many years before the onset of disease. Indeed, for disorders of incomplete penetrance, it is possible that an embryo carrying a mutation might never develop the associated disease. Some of the cancer predisposition syndromes that have been screened in embryos fall into the late onset/incomplete penetrance categories, although the probability of developing disease at some stage of life is generally high (>80%). Another objection to PGD for cancer predisposition has been that treatment options are sometimes available for affected individuals. However, treatment usually involves surgery and can be of a fairly radical nature, risking a significant impact on the patient's quality of life (e.g. bilateral mastectomy and removal of the ovaries in patients with a BRCA mutation). In the case of HLA typing, the greatest concern is that a new individual is being created, at least in part, for the benefit of another. Additionally, stem cell transplantation is not always successful. If it fails, will the new child then be subjected to bone marrow extraction procedures, an altogether more risky procedure? If the affected sibling dies, how will that affect the family dynamic and the new child's place within it? These questions and others continue to be debated.

Currently, there is no global consensus on what embryo testing should be permissible and what should not, and given cultural and religious differences, this

situation is unlikely to change. Some countries (e.g. Germany) have instituted highly restrictive laws governing the use of PGD, while others have adopted a more liberal approach. In some cases the more permissive models are nonetheless tightly regulated, operating within a well-defined legal framework (e.g. the UK), while others have relatively little oversight from the state or professional bodies (e.g. the United States).

PGD today: transport PGD and efficacy

In the early days of PGD, many IVF clinics set up their own diagnostic laboratories or established close links with academic labs specializing in genetics, allowing them to begin offering PGD. This growth in PGD was driven, in varying degrees, by a mixture of academic interest, desire to offer an important new medical service and commercial forces. The ability to provide PGD has often been seen as an indicator of an IVF clinic's technological advancement and consequently there have been pressures on laboratories to list PGD testing amongst their repertoire of services. In some respects PGD has become increasingly routine, yet it remains a complex procedure, requiring a controlled environment, expert staff trained in genetics and expensive equipment and reagents. In order to cover the high costs associated with PGD, it is necessary to conduct large numbers of cycles each year, which is beyond the reach of all but the very largest IVF clinics. For this reason, few clinics still maintain their own PGD laboratory, most preferring to send the samples (polar bodies, blastomeres or trophectoderm biopsies) to specialist PGD laboratories that receive cases from multiple IVF clinics. This strategy has been termed 'transport' PGD. The transport PGD model allows PGD to be offered at lower prices and is associated with good rates of accuracy and pregnancy [9]. Using a transport PGD approach, any IVF clinic can offer PGD provided they have staff trained in embryo biopsy procedures. Results are not affected by the distance the biopsied material has travelled. Since transport PGD allows patients to undergo treatment at a local IVF clinic, the stress and expense of travelling to distant clinics with an in-house PGD capability can be eliminated.

Accuracy rates using PGD typically exceed 95%. Indeed, most PGD protocols for single gene disorders are predicted to have accuracies of approximately 99%. Despite these impressive figures, some clinical geneticists and genetic counsellors have been reluctant to discuss the option of PGD with patients. The principal reasons for this have been the high cost of the procedures and the perception that the chances of having an unaffected child are poor, due to the relatively low pregnancy rates achieved using IVF. However, this viewpoint is largely based on data from the early years of PGD. In the last decade, IVF success rates have continued to improve, while PGD costs have tended to remain static or even to fall. Thus a re-evaluation of the role of PGD seems overdue. Analysis of outcome data from one laboratory offering PGD services (Reprogenetics), conducted three years ago, revealed that almost half of the patients undergoing their first PGD cycle achieved a pregnancy [9]. That figure now stands at 60%.

Future developments

The number of patients undergoing PGD continues to increase each year, indicating that this reproductive strategy is likely to grow in importance in the coming years. If IVF success rates undergo further improvements, as is likely, PGD will become an increasingly attractive option for many couples at high risk of transmitting a serious inherited disorder. In addition to IVF efficiency, another important consideration is the cost of the procedure. At present, PGD is expensive, adding several thousand dollars to the price of an IVF cycle. However, research into low-cost PGD strategies is underway and may soon permit testing for some diseases to be carried out at a fraction of current costs. This will be particularly important if PGD is to be more widely applied in developing countries, many of which have exceptionally high carrier frequencies of specific single gene disorders (e.g. sickle cell anemia in West Africa and thalassaemias in South East Asia).

A problematic area for PGD has been the amount of customization necessary for each case. This has sometimes resulted in lengthy delays to treatment and greatly increases the costs involved. Testing of unique chromosome rearrangements has become much easier since the introduction of array-CGH, which provides a single platform applicable to the majority of chromosome rearrangements. However, most single gene PGD cases still involve substantial patient-specific protocol design and work-up. Linkage-based approaches, including PGH, have helped reduce the amount of customization in some cases, but also have limitations.

A more radical change in PGD methodology has involved the use of whole genome amplification and microarray technology, allowing simultaneous

analysis of large numbers of polymorphisms scattered throughout the genome. In most cases the polymorphisms are variations in the DNA sequence involving a substitution of one nucleotide for another at a single site (single nucleotide polymorphisms – SNPs). Parents and embryos are tested using microarrays, providing results for thousands of SNPs distributed along the length of each chromosome. Using this approach, sometimes referred to as karyomapping, it is possible to track the inheritance of every segment of every chromosome from parent to child, including all regions of the genome containing genes associated with human disease [11] (Fig. 33.3). Thus, karyomapping provides a universal method for PGD of any single gene disorder via linkage analysis. Additionally, since the inheritance of individual chromosomes can be followed, many forms of aneuploidy can also be detected (e.g. trisomy caused by the inheritance of three genetically distinct copies of the same chromosome). The simultaneous diagnosis of chromosome abnormality and single gene disorders has previously been problematic for PGD, as the principal technique used for chromosome testing (FISH) and the method underlying single gene diagnosis (PCR) cannot be reliably applied to the same cell. Although methods such as karyomapping may represent the future of PGD, they are currently expensive and do not fit comfortably into the highly restricted timeframe available for embryo diagnosis. Further optimizations will be required before they are widely applied.

Karyomapping is one of several new PGD methods, including array-CGH, which utilize whole genome amplification (WGA) technology. Unlike PCR, which confines amplification to a single (or small number) of defined sequences, WGA aims to amplify all regions of the genome. Using WGA, sufficient DNA is produced from a single cell to permit types of genetic analysis that generally require samples composed of thousands of cells. While utilization of powerful genetic methods holds great promise for the future of PGD, ethical concerns have been raised over the vast quantity of information provided. For example, it will become increasingly possible to test individual embryos for large numbers of different diseases and predispositions simultaneously. Additionally, it will soon be technically possible to assess characteristics influenced by the combined effects of multiple genes (polygenic traits). Some degree of independent oversight or legislation may be necessary in order to control the use of emerging PGD technologies and to ensure that the potentially sensitive data produced is safeguarded.

Concluding summary

The efficacy of preimplantation genetic diagnosis is inseparably linked to the efficiency of IVF treatment. As IVF success rates continue to improve, so the attractiveness of PGD to patients at high risk of transmitting an inherited disorder will also increase. With new technologies increasing the accuracy, availability and scope of PGD and other innovations reducing costs, it seems likely that PGD will become an increasingly important reproductive strategy in the future.

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Chapter 34

Preimplantation genetic screening

Dagan Wells

The frequency and impact of aneuploidy

It is well established that chromosome abnormality (aneuploidy) is common during the early stages of pregnancy in humans. A high prevalence of aneuploidy is evidenced by the frequent detection of cytogenetically abnormal gestations following prenatal testing using chorionic villus sampling (CVS) or amniocentesis. Abnormalities affecting chromosomes 13, 18, 21 and the sex chromosomes (X and Y) are particularly common and show increasing prevalence with advancing maternal age. Although aneuploid conceptions can occasionally result in a live birth (e.g. trisomy 21, Down syndrome), the vast majority are incompatible with development to term. In addition to the chromosomes mentioned above, trisomies (three copies instead of the usual two) affecting chromosomes 15, 16 and 22 are frequently observed in tissue from miscarriages. It is estimated that more than two-thirds of all first trimester miscarriages are a consequence of aneuploidy, underscoring the lethality of chromosome imbalance [12, 15].

While it has long been clear that incorrect numbers of chromosomes are relatively common during early pregnancy, molecular cytogenetic data accumulated over the last two decades has revealed that abnormality rates are higher still at the very earliest stages of development. During the 1990s, multiple studies using fluorescent *in situ* hybridization (FISH) revealed that the majority of human preimplantation embryos contain aneuploid cells (e.g. 3, 16). Analysis of oocytes and/or their corresponding polar bodies yielded similar data, indicating a maternal origin for most of the embryonic abnormalities [24]. Recently, more powerful cytogenetic methods (e.g. microarrays, discussed in greater detail below) have confirmed much of the FISH data, demonstrating aneuploidy rates of over 25% for the oocytes of women below 35 years of age and rates in excess of 75% for women over 40 [5].

Preimplantation genetic screening – PGS

The high prevalence and lethality of aneuploidy means that, inevitably, many of the embryos produced during a typical IVF cycle will carry fatal genetic flaws. It has been suggested that IVF success rates could be improved if chromosomally normal embryos could be identified and prioritized for uterine transfer, an approach known as preimplantation genetic screening (PGS). In theory, avoiding the transfer of aneuploid embryos should lead to improved pregnancy rates and reduced incidence of miscarriage and Down syndrome. However, achieving selective transfer of genetically 'normal' embryos is not straightforward, as the standard methods of embryo viability assessment used in IVF laboratories, based upon morphological characteristics, do not reveal aneuploidy. Chromosomal abnormality has very little effect on the appearance of embryos, especially prior to activation of the embryonic genome, which occurs around the 4–8 cell stage [1]. For this reason, additional PGS tests, aimed at providing cytogenetic information, have been devised.

Currently, chromosomal information can only be obtained by sampling genetic material from the oocyte or embryo. In the case of oocyte testing, the first (and in some cases second) polar body is biopsied. The polar bodies represent the reciprocal products of each meiotic division, such that a chromosome gain in a polar body typically indicates a chromosome loss in the corresponding oocyte. Historically, embryo analyses have usually been undertaken at the cleavage stage, with one or two blastomeres removed on day 3.

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However, analysis at the blastocyst stage, involving biopsy of a small amount of trophectoderm tissue (usually ~5 cells), is becoming increasingly popular. Regardless of the type of biopsy, the zona pellucida must first be breached to allow access to the cells inside. Currently, this is usually achieved using a laser, although in the past a weak acid solution (acid Tyrodes) was commonly used to dissolve a hole in the zona pellucida prior to cleavage stage biopsy. Mechanical dissection of the zona pellucida was typical for polar body biopsy.

PGS using FISH

Initially, PGS strategies involved the use of FISH applied to cells (polar bodies, blastomeres or trophectoderm) that had been fixed on microscope slides. The FISH technique involves the labelling of specific chromosomal regions with fluorescent DNA probes, depositing a discrete coloured spot on each of the chromosomes tested (Fig. 34.1). Unlike traditional cytogenetic methods, which rely upon the visualization of metaphase chromosomes, FISH allows enumeration of chromosome copy number during any phase of the cell cycle. Even at interphase, when chromosomes are diffuse, overlapping, and contained within the nucleus, distinct FISH 'signals' can be detected and counted.

Data from multiple prospective trials suggested that biopsy of polar bodies or blastomeres, followed by embryo selection based upon the results of FISH analysis, led to improvements in a variety of IVF outcomes, such as reduced miscarriage rate and enhanced implantation rate [8, 17]. These encouraging results fuelled a rapid growth in the utilization of PGS. However, when randomized trials were finally, belatedly, carried out, it was found that PGS performed at the cleavage stage provided no significant benefit [11, 14]. Given the high incidence of aneuploidy in human embryos and the detrimental effect of such abnormalities, the fact that PGS had apparently failed to improve IVF success rates surprised many in the field. The reason for the poor results is likely to be a combination of both technical and biological factors [18]. Most FISH protocols assessed less than half of the chromosomes, making it inevitable that some aneuploid embryos would be incorrectly assigned a 'normal' diagnosis. Additionally, mitotic chromosome segregation errors, arising after fertilization of the oocyte, are common during the preimplantation phase of development and sometimes produce 'mosaic' embryos, composed of a mixture of normal and aneuploid cells. Mosaicism of this type presents problems for diagnosis based upon analysis of a single blastomere, as the cell tested might not be representative of the remainder of the embryo.

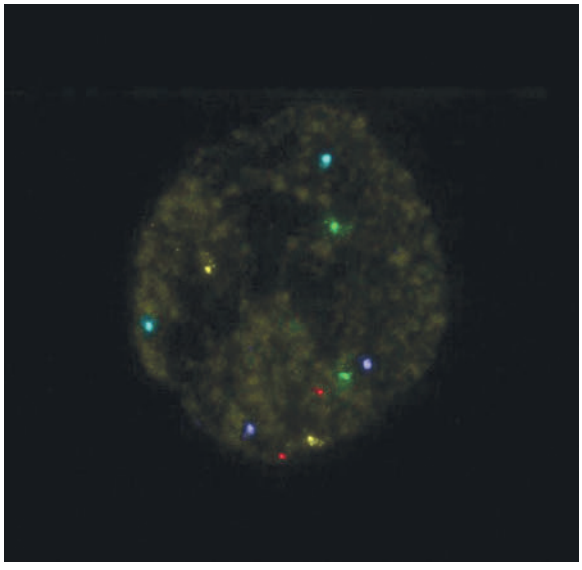

Figure 34.1 Fluorescent *in situ* hybridization analysis of a single blastomere biopsied from a cleavage stage embryo. Five different chromosomes were enumerated using chromosome specific probes labelled with fluorochromes of different colours: 13 (red); 21 (green); 22 (yellow); 16 (cyan); 18 (blue/violet).

A new generation of chromosome screening methods – microarray CGH

The negative results of randomized controlled trials persuaded many to abandon PGS, but some groups argued that the trials had utilized suboptimal methods and remained convinced that PGS could yield benefits if performed by experienced teams using appropriate techniques. This debate, which has at times been heated, cast a shadow over the field of preimplantation genetic diagnosis for several years. However, the emergence of new technologies that overcome the limitations of FISH may finally end the argument. Several methods allowing comprehensive chromosome screening have recently been described. The methods do not require cells to be spread on microscope slides, eliminating one of the most technically challenging aspects of FISH-based PGS methods and allow detection of aneuploidy affecting any chromosome with

high accuracy, thus providing a solution to the principal diagnostic limitation of earlier techniques [25].

The most widely used method for comprehensive chromosome screening of oocytes and embryos is microarray comparative genomic hybridization (also known as array-CGH or aCGH). In this case, the biopsied cell(s) is amplified using a whole genome amplification technique; the DNA is then labelled with a fluorescent dye and applied to a microarray comprising numerous probes composed of DNA complementary to specific chromosomal regions affixed to a solid support (e.g. a glass slide); a 'reference' DNA sample derived from a chromosomally normal individual, labelled in a different fluorescent colour, is applied to the microarray at the same time. Detection of loss or gain of chromosomal material involves computer assisted analysis of the colour of each probe on the microarray. An equal quantity of fluorescence corresponding to the sample DNA (i.e. polar body, blastomere or trophectoderm biopsy) and the reference DNA (i.e. normal karyotype sample) is indicative of normality for the chromosomal region corresponding to the probe; a gain of chromosomal material is revealed by an excess of fluorescence attributable to the sample; a deficiency of fluorescence from the sample relative to the reference DNA demonstrates a loss of chromosomal material (Fig. 34.2). This approach has the potential to provide a highly accurate analysis of every chromosome. However, most commercially available microarray platforms are not suitable for single cell analysis, so it is advised to only use those that have been validated at the single cell level. Multiple studies have now been conducted in order to assess the performance of aCGH, demonstrating that it provides an accurate evaluation of the chromosomes in 94% of polar bodies, 97.1–98.8% of blastomeres and 97% of trophectoderm biopsies [9; 7; Reprogenetics unpublished data).

Alternative methods for comprehensive chromosome assessment

Although aCGH has become the dominant method for PGS, other alternatives do exist. The most notable of these are quantitative PCR and single nucleotide polymorphism (SNP) microarrays. Quantitative PCR involves the use of a single, massive, multiplex PCR, employing a large number of individual primer pairs to simultaneously amplify multiple defined DNA fragments. Aliquots of this mixture are then taken and each locus is subjected to further amplification, this time individually rather than in a multiplex reaction. The accumulation of amplified DNA is monitored in real time during this second round of PCR (real-time PCR), which allows the number of copies of the locus to be accurately quantified. Aneuploidy is assessed by measuring the amount of DNA amplified from several loci on each chromosome, compared with the quantity of DNA produced by amplification of the same loci in a chromosomally normal sample.

Publications indicate that quantitative PCR provides a high accuracy diagnosis of aneuploidy when applied to trophectoderm biopsies (in excess of 98%) [23]. The major advantage of quantitative PCR over other PGS strategies is its speed. Diagnosis can be completed within 4 hours (less than half the time required by the fastest aCGH protocols), making it

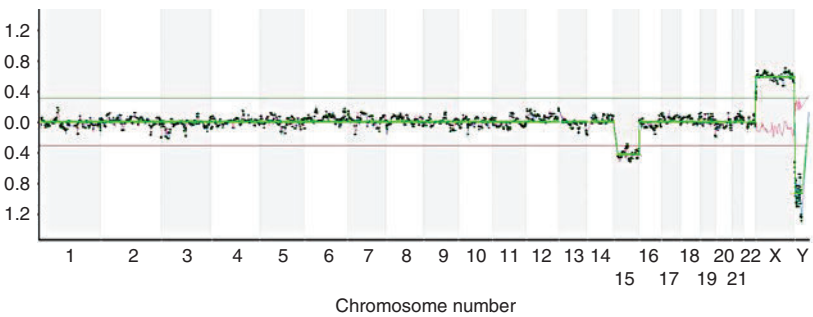

Figure 34.2 Microarray comparative genomic hybridization analysis of a trophectoderm biopsy. Data from each probe is represented as a discrete spot, arranged in the same order that the probes appear along the length of each chromosome. Probes that have hybridized an equal amount of test (embryo) DNA and reference (normal) DNA have a value of ~0.0. Probes derived from chromosomal regions present in a trisomic state have values >0.3, whereas monosomic regions are indicated by values < 0.3. The embryo shown is female (more X-chromosome DNA and less Y-chromosome DNA compared with a male reference) and is affected by monosomy 15.

particularly attractive for diagnosis at the blastocyst stage, when the time available for testing is extremely limited. The main limitations of the method are a lack of scalability (several real-time PCR instruments may be needed if multiple cases are to be processed on the same day) and the fact that the accuracy of the method may be reduced if it is used for the analysis of single cells (e.g. polar bodies or single blastomeres). However, for analysis of blastocysts the approach works well and has been associated with excellent clinical outcomes, including significantly elevated implantation and pregnancy rates in a recent randomized trial [5, 20].

Another strategy for comprehensive chromosome screening is the use of SNP microarrays, an approach that involves tracking the inheritance of polymorphisms from the parents to their embryos [2, 10, 13, 21]. Samples of DNA are collected from each of the parents and analyzed using a microarray that simultaneously evaluates many thousands of polymorphisms scattered throughout the genome. The polymorphisms are variations in the DNA sequence affecting a single nucleotide. A huge number of such SNPs exist in the human genome and in almost all cases there are just two distinct sequence possibilities (i.e. two alleles) at each site. Each of the parental chromosomes has a unique combination of SNP alleles, analogous to a DNA fingerprint, providing a means of confirming whether a particular chromosome is present or absent from a sample.

The process of PGS involves cell biopsy, followed by whole genome amplification, fluorescent labelling of the amplified embryo DNA and ascertainment of SNP genotypes using a microarray. The results from the embryos are compared with data obtained from the mother and father and an attempt is made to deduce which of the parental chromosomes have been inherited by each embryo. The process of defining the sequence of alleles along the length of each chromosome and following their inheritance from parent to embryo is sometimes referred to as karyomapping [10]. Using SNP microarrays a chromosome loss or gain may also be indicated by a respective decrease or increase in the fluorescence intensity of all the microarray probes on the affected chromosome. Additionally, monosomy is revealed by apparent homozygosity for all SNPs on the affected chromosome.

The use of SNP microarrays for PGS has the advantage that the parental origin of aneuploidies can sometimes be inferred. Although the vast majority of abnormalities are of maternal origin, rare couples suffer an unusually high level of aneuploidies derived from sperm. Information on parental origin may be useful for such couples, especially if donor gametes are being considered. The large number of polymorphisms assessed by SNP arrays also provides a DNA fingerprint for the embryos tested. Clinically, fingerprinting may allow extremely rare cases of embryo/gamete mix-ups to be detected, since the SNP alleles detected in the embryo should match those of the couple undergoing IVF. However, the principal value of DNA fingerprinting may be scientific. If a child is born following an IVF cycle in which two embryos were transferred, it is usually unclear which of the embryos produced the pregnancy. However, if the embryos were fingerprinted prior to transfer, the results can be compared to those obtained from the resulting child, revealing which embryo was responsible for the pregnancy [21]. Essentially, this allows direct comparison of the viability of embryos from the same couple and the same cycle after simultaneous transfer to the same uterus.

Not only can SNP microarrays provide information about the inheritance of specific parental chromosomes, they are capable of revealing information about the origin of relatively small regions of each chromosome (of the order of a few million base pairs). This makes it possible to track the inheritance of specific loci associated with monogenic disorders. For example, a couple in which the male and female are both carriers of a cystic fibrosis mutation could use SNP microarray analysis of their embryos to reveal which copies of chromosome 7 (and more specifically region 7q31, the site of the cystic fibrosis gene) each had inherited. Thus, SNP microarrays potentially allow diagnosis of single gene disorders for some patients as well as aneuploidy screening.

The main drawbacks of PGS using SNP microarrays are that tests are more expensive than aCGH or quantitative PCR and that current protocols take longer to perform than alternative PGS methods. Although it is possible to complete SNP analysis within 24 hours, this requires laboratory staff to work into the evening. Recent research data suggest that future optimizations will result in accelerated protocols and reduce costs, but at the time of writing more work is still needed.

Several new methods of chromosome screening are under development and hold great promise for the

future. Of these techniques the most important is likely to be next generation sequencing (NGS), which has the power to provide quantitative data (e.g. chromosome copy number) and also information about DNA sequence (e.g. diagnosis of single gene disorders, embryo fingerprinting, analysis of parent of origin for aneuploid chromosomes). Preliminary evaluation carried out in our laboratory suggests that NGS can provide a highly accurate assessment of aneuploidy in single cells in less than 24 hours and at lower cost than any of the methods currently used for PGS. The cost of each test is an extremely important consideration for PGS as a single patient will usually produce multiple oocytes/embryos and therefore require several tests, multiplying the cost of the screening technique.

PGS after FISH: do the new technologies really work?

The accuracies of all of the new approaches discussed above appear to be high (94–99%), as confirmed by studies of embryos donated for research in which each embryo could be tested several times and the test results assessed for concordance. However, accuracy is of little relevance if the test does not deliver the anticipated improvements in IVF outcome. One of the first studies suggesting a significant improvement in IVF outcome following comprehensive chromosome screening utilized CGH applied to embryos at the blastocyst stage [19]. An implantation rate of 68.9% was achieved (more than two-thirds of embryos diagnosed euploid and transferred produced a child). This result was particularly remarkable given the fact that the mean maternal age was 37.7 and that most of the patients had a history of unsuccessful IVF treatments. The study was not randomized, but did employ a very well matched control group of patients from the same clinic undergoing treatment at the same time. The implantation rate in the control group was also very good (44.8%), yet still 50% lower than achieved after screening ($p< 0.0001$). This study reignited interest in PGS and has been followed by several other encouraging studies.

One investigation, demonstrating the diagnostic power of chromosome screening, involved biopsy of a single blastomere from cleavage stage embryos or several trophectoderm cells from blastocysts, followed by transfer of embryos before genetic analysis was carried out. Retrospective data analysis revealed that 99 aneuploid embryos had unknowingly been transferred to patients. It was found that only 4% of these embryos had produced a viable pregnancy, 94% failing to implant and the remaining 2% miscarrying. Thus, PGS using this method (a SNP microarray) was shown to have a 96% negative predictive value [23].

Although there are increasing data from prospective studies indicating that new comprehensive chromosome screening methods provide improved embryo selection and enhance IVF outcomes, significant scepticism concerning the efficacy of PGS still remains and only high-quality data from randomized controlled studies are likely to be accepted as proving the validity of the new PGS strategies. Fortunately, several randomized trials are already underway to assess the efficacy of comprehensive chromosome screening applied to oocytes and embryos. Interim data analysis from several studies suggests a positive impact of PGS using aCGH and quantitative PCR at the cleavage and blastocyst stages, with higher implantation rates and reduced incidence of miscarriage contributing to an improved birth rate per cycle. However, most of these studies have not yet been completed and the data has not been subjected to rigorous review. The only randomized controlled trial with published, peer-reviewed data, concerns the use of aCGH to screen blastocysts produced by good prognosis patients (< 35 years) [26]. Depending on the group into which the patient had been randomized, embryos were prioritized for transfer either on the basis of a traditional morphological assessment or on the basis of chromosomal normality. Embryo biopsy was carried out on day 5 and all transfers were carried out on day 6. The ongoing pregnancy rate (≥20 weeks gestation) per cycle started was 41.7% for the control group versus 69.1% for the patients whose embryos were screened using aCGH ($P< 0.01$). The sustained pregnancy rates per cycle were particularly impressive given that all cycles involved elective single embryo transfer [26].

If further studies conclusively prove that PGS leads to improved pregnancy rates per cycle, as well as reduced miscarriage rates and a lower risk of Down syndrome, there will be a strong argument for expanding the utilization of the technology. At present, it is estimated that PGS is used in fewer than 5% of IVF cycles worldwide. Traditionally, PGS has been applied to restricted groups of patients, specifically those of advanced reproductive age (women in their late thirties or forties), couples who have suffered several miscarriages, patients with a history of unsuccessful IVF

treatments and those who have experienced previous aneuploid pregnancies. These patients often produce high levels of aneuploid embryos and consequently it is not uncommon for all the embryos tested within a cycle to be diagnosed abnormal. While a result of this type may provide clinically useful information (potentially avoiding further miscarriages or providing an explanation for unsuccessful IVF attempts), it does not help to achieve the pregnancy that the patients desire. It may turn out that classes of patient that have not previously been offered chromosome analysis could benefit the most from PGS.

Women under 35 years of age frequently produce several blastocysts and are often considered to be candidates for elective single embryo transfer. However, such patients are rarely offered PGS, as their risk of aneuploidy has been considered to be low. In recent years, multiple studies have proven that this is not the case. Comprehensive chromosomal analysis using aCGH has shown that more than a third of blastocyst stage embryos from young patients are aneuploid, indicating a significant risk that the embryo chosen for transfer will fail due to a chromosomal abnormality [6]. Embryos from women under 35 years of age have an appreciable aneuploidy rate, but because they usually produce several blastocysts, it is almost always the case that at least one euploid embryo is found. Cycles with no transfer are rare and pregnancy rates in this patient group have been reported to be extremely high following PGS [19, 20, 26].

Modern methods of aneuploidy screening appear to have overcome many of the deficiencies of earlier techniques, allowing comprehensive analysis of chromosomes to be undertaken with high accuracy. The improved implantation rates and reduced incidence of miscarriage provided by a successful PGS method will lead to reductions in the average length of time a patient must wait before obtaining a viable pregnancy. The number of transfer procedures to which a woman undergoing IVF is subjected will be reduced, since the risk of transferring non-viable embryos is lessened. Additionally, aneuploid embryos are not cryopreserved and consequently fewer embryos need be frozen, saving patients' money and providing them with a more realistic assessment of the potential of their banked embryos. It is likely that more than half of all embryos currently stored worldwide are genetically abnormal and have no possibility of producing a child. Effective PGS protocols will also yield valuable prognostic information concerning the proportion of viable embryos produced per cycle, assisting in patient management and counselling, especially towards the end of the female reproductive life span. While well-controlled clinical studies assessing the potential of modern PGS techniques remain few at this time, many are in progress, and early data provide reason for cautious optimism. It is hoped that the controversy over the use of PGS may soon be over and the true value of this exciting strategy will be better defined.

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Chapter 35

The biology and therapeutic potential of embryonic stem cells

Richard Gardner

Introduction

Embryonic development is typically characterized not only by a spectacular, spatially ordered diversification of cells, but also a dramatic increase in their number. Even when final adult size has been reached, the demand for new cells remains remarkably high, largely to make good losses through shedding from the surface of the skin and intestine and destruction within the haematopoietic system. According to one estimate, the entire body weight in cells is turned over during the course of a year. The critical players in ensuring that such losses are made good are 'stem' cells, cells that retain the ability to produce progeny identical in proliferative potential and other cogent properties to themselves as well as more overtly differentiated descendants. The repertoire of differentiation in which their descendants engage varies according to their source, a fact that is acknowledged by a common system for their classification. Thus, they are referred to as uni-, bi-, multi- or pluri-potent, according to whether they yield one, two, many, or most types of differentiated progeny. While the terms 'pluripotent' and 'totipotent' are often used interchangeably, the latter should strictly be applied only to cells that retain the ability to form a new individual unaided including, in the case of mammals, all extraembryonic membranes as well as the fetus itself; in other words, produce the entire conceptus. Thus far, the only cells that have been shown to exhibit totipotency are blastomeres isolated from early cleavage stages.

Recent years have witnessed a dramatic increase in interest in cells with stem cell characteristics, particularly those exhibiting pluripotency. This has been driven largely by two considerations. One was to be able to modify genetically in an incisive way cells that retain the ability to colonize the germline. The aim here was to greatly accelerate the process of elucidating the role of specific genes, a task that had depended hitherto on studying spontaneous or induced mutations in whole organisms. The other was to exploit the potential of stem cells clinically as a source of tissue for repair or restoration of body parts. Yet another source of interest in these cells is the notion that cancer is rooted in their aberrant behaviour, a concept of some antiquity that is currently enjoying a renaissance.

The quest for pluripotential stem cells

Interestingly, it was a comparative study of benign tumours called teratomas and their highly malignant counterparts termed teratocarcinomas, which provided the first clear evidence for the existence of pluripotent stem cells. The common feature of both types of tumour is that they typically contain a much richer variety of types of differentiated cells than any others, including those representatives of all three classical embryonic germ layers. The uniquely rich range of differentiation shown by these tumours is a consequence of their originating either from male or female germ cells, or from early embryos. While human teratocarcinomas had been an object of study for generations, mainly from a pathological perspective, it was the humble laboratory mouse that afforded the clearest insight into their biology [1]. Thus, selective mating in the inbred 129 strain yielded a sufficiently high incidence of teratomas and teratocarcinomas to enable their origin to be traced back to unscheduled growth of occasional spermatogonial stem cells within the seminiferous cords of the early fetal testis. In inbred LT mice, early embryos could be found in the oviducts and uteri of young adult females that had never been exposed to males, demonstrating that ovulated eggs

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

were routinely engaging in parthenogenetic activation. By 6 months of age, a substantial proportion of females of this strain have teratomas or teratocarcinomas in one or both ovaries, implying that oocytes were embarking on a form of development even before they were ovulated. The majority of such germ cell tumours could be classified as teratomas since they did not show sustained growth in their primary host and failed to 'take' on transplantation to fully histocompatible secondary ones. The remainder were, however, classed as teratocarcinomas because they were not only capable of growing progressively in the primary host but could be passaged indefinitely through a succession of further histocompatible mice. Hence, it became clear that what distinguished teratocarcinomas from teratomas was the presence of stem cells with a seemingly limitless capacity for self-renewal. The problem was that although candidate morphologically undifferentiated cells could be seen in histological preparations of teratocarcinomas, they were too dispersed to offer much prospect of enriching them for critical study. A vital breakthrough came with the conversion of the solid tumours to 'ascites' form by injecting small fragments of them into the peritoneal cavity. This resulted in the production of tiny solid balls of cells termed simple embryoid bodies (EBs), with a core of uniformly undifferentiated-looking cells surrounded by a rind of differentiated endodermal type cells (Fig. 35.1). These EBs could either reproduce themselves by a process of budding or engage in a process of differentiation involving cavitation and cell rearrangement, during the course of which the morphologically undifferentiated core cells disappeared (Fig. 35.1). Most interestingly, even when transplanted singly or in small numbers to histocompatible hosts, simple EBs proved highly malignant, while their cavitated or 'cystic' counterparts did not. This finding strongly endorsed the view that the malignant stem cells resided in the core of simple EBs and were lost when these became cystic. This was tested in a heroic experiment in which 1788 grafts of single core cells were made to histocompatible adult mice. While one of the 44 resulting tumours contained only extraembryonic tissues, all the remainder were teratocarcinomas, many of which included as rich a diversity of cell types as the parent tumour from which the single transplanted cells were obtained. This established beyond doubt that the perpetuation of murine teratocarcinomas was due to the persistence of a pluripotential type of stem cell to which the term embryonal carcinoma (EC) cell was applied. What engendered particular interest in EC cells was the demonstration by three laboratories that they could participate in normal embryonic development following injection into blastocysts [2]. However, excitement was soon tempered because the resulting chimaeric offspring not infrequently developed tumours that, regardless of type, were invariably of EC origin. Moreover, the cells often proved to be abnormal chromosomally and, most disappointingly, a claim that they could colonize the germline and might thus be exploitable for deriving genetically modified mice, could not be confirmed [3]. The suspicion that all such shortcomings of these cells might relate to the lengthy process of their derivation in vivo prompted attempts to short-circuit this by explanting blastocysts into enriched medium on growth-inactivated feeder cells, conditions designed to maintain proliferation of inner cell mass cells in an undifferentiated state. This strategy yielded pluripotent stem cells that were greatly superior to EC cells in retaining karyotypic normality, participating in tumour-free chimaera formation and, most importantly, being routinely capable of forming gametes [4–6]. Such embryonic stem cells (ESCs) are now well established as a vital tool in transgenesis studies designed to elucidate the functions of specific genes, a burgeoning number of which have now been investigated in this way.

The clinical potential of ESCs

Pluripotency rather than specific competence to colonize the germline was the prime consideration in arousing interest in the possibility of obtaining the human counterpart of murine ESCs. If successful,

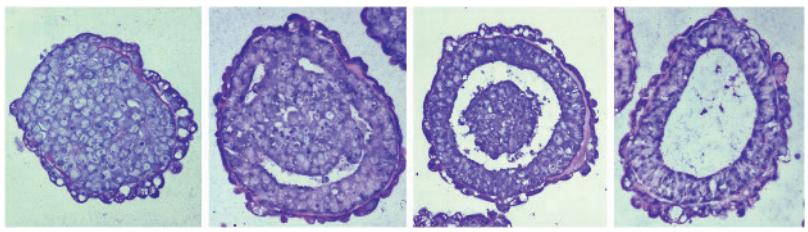

Figure 35.1 Stages in transformation of a teratocarcinoma-derived simple embryoid body (left) into a bilaminar cystic one (right) by a process akin to embryonic gastrulation (from E. Coucouvanis and G. M. Martin, *Cell* **83** (1995): 279–87. Courtesy of Professor Gail Martin).

such cells could potentially serve as a source of most if not all types of differentiated cells required for regenerative medical purposes [7]. Availability of human material for deriving such stem cells was not an issue since assisted conception in vitro invariably entails the production of many more early embryos than are needed for infertility treatment. It was Robert Edwards, the original pioneer of human IVF, who was the first to suggest as long ago as 1982 that such 'spare' embryos might be used in this way. Inexplicably, it took well over a decade before this suggestion was taken seriously and the first human ES cells lines were produced.

The biology of ESCs

Attempts to derive pluripotential stem cells from early embryos have now been made in a wide range of mammals, as also in several other vertebrate species (Table 35.1). So far, however, murine rodents have provided the only source of 'true' ESCs, i.e. pluripotential cells that have been shown unequivocally to include functional gametes as well as all types of adult somatic cells in their repertoire of differentiation. Critical testing of the potency of rodent ES cells was made possible through their ability routinely to colonize the epiblast, the tissue from which they originated, following their reintroduction into preimplantation embryos (Fig. 35.2). Nevertheless, even in the mouse, there are marked strain differences in the facility with which ESCs can be obtained. While in non-rodent species, morphologically undifferentiated stem cells obtained from blastocysts have been shown to be able to produce somatic cells representative of all three germ layers, in no case have functional gametes yet been obtained. Moreover, typically in such species the case for pluripotency has depended principally on assessing the types of cells found in teratomas obtained by grafting colonies of undifferentiated cells ectopically in histocompatible or immunosuppressed adult hosts. This is because extensive colonization of preimplantation embryos has not been achieved or, specifically in the case of the human, attempting to do so would obviously be unacceptable ethically. This teratoma assay can, however, prove misleading because what is obtained tends to vary according to the host site that is chosen for grafting. In addition, specifically in the case of the human, one has no option but to do xenografting, typically to mice that are immunologically compromised genetically. Hence, pluripotential stem cells derived from preimplantation stages in these other species should properly be termed 'ESC-like' stem cells (ESLSCs) to distinguish them from true ESCs.

Table 35.1 Species other than mouse in which derivation of ESCs has been attempted

Rat*	Marmoset
Golden hamster	Rhesus monkey
Rabbit	Human
Mink	Chicken
Pig	Medaka
Sheep	Zebrafish
Cow	Gilthead bream
Buffalo	Asian Sea Bass
Horse	

*Only species other than mouse from which germline-competent ESCs have been obtained.

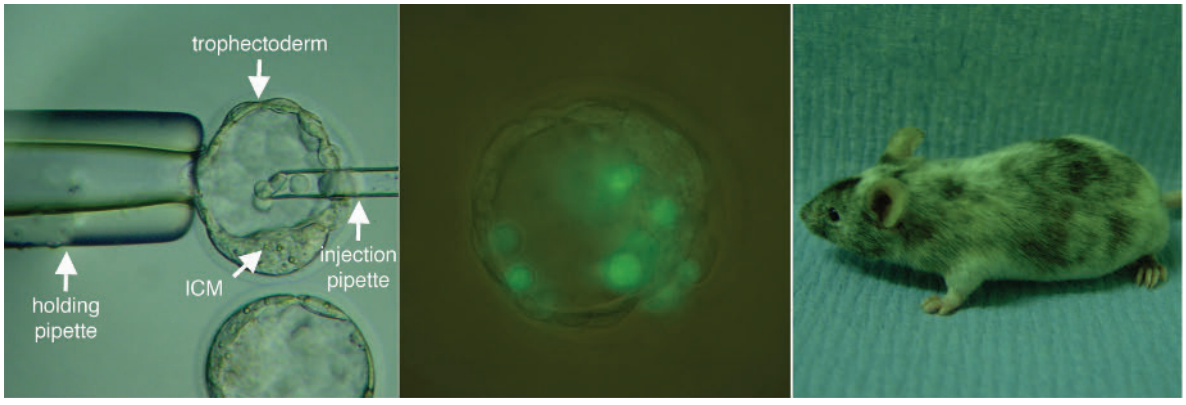

Figure 35.2 Injection of murine ESCs into blastocyst of albino strain (left), distribution of fluorescently tagged ESCs within blastocyst (centre), and a typical resulting chimaera showing extensive donor coat pigmentation (right). (Courtesy of Dr Paul J. Tesar)

Murine ESCs have been found to originate exclusively from the epiblast, the deep tissue of the ICM of the late blastocyst, and lines of them have been obtained from single isolated cells of this tissue. Hence, interaction with other cells, a so-called community effect, is clearly not necessary for their production which seems to be a property common to most if not all cells within the tissue. The possibility of obtaining stem cells from earlier cleavage stages with the potential to produce extraembryonic as well as all fetal cell types has been explored in several studies. While true ESC lines have successfully been derived thus, they appear to be indistinguishable from those of epiblast origin in both potency and other attributes. Hence it would seem likely that cells have to progress to the point of entering the epiblast lineage before becoming susceptible to ESC derivation. Moreover, susceptibility is limited to a brief initial period of existence of the epiblast during the late blastocyst stage. Thus, although pluripotential cells can be obtained readily from early postimplantation epiblast, these clearly differ from true ESCs both in their requirements for sustained growth in an undifferentiated state, and their inability to yield chimaeras on transplantation into preimplantation embryos. As discussed later, where comparisons have been made, the properties of these later originating so-called epiblast stem cells (EpiSCs) are similar to those of ESLSCs obtained in other species [8, 9]. While at its foundation, the epiblast consists of a solid ball of unpolarized cells, it rapidly undergoes reorganization to form a pseudostratified epithelium, which might account for the early loss of ability of its cells to colonize the preimplantation embryo.

Conditions required to maintain murine ESCs in an undifferentiated state have been the subject of much research. This has led to the association of a number of genes with pluripotency, and strenuous efforts to define the conditions required to maintain their expression. Key players include Oct4, Nanog, Rex1 and Sox2. However, before discussing the principal findings, it is relevant briefly to consider the special nature of the mitotic cycle of these cells. The typical somatic cell cycle is controlled by regulating progression through G1 and entry into S phase, processes that are orchestrated by predictable cyclic changes in the expression of many genes or in post-translational modifications of their encoded proteins, for example in the extent of phosphorylation. The retinoblastoma protein (RB) and its relatives (p107 and p130) are key components governing the G1/S transition. In their hypophosphorylated state, such proteins inhibit expression of genes required for entry into S by sequestering transcription factors of the E2F family. Progress from G1 depends on RB and related proteins being phosphorylated sequentially by a complex of cyclins coupled with cyclin-dependent kinases (cdks). Phosphorylation of these proteins by cyclinD/CDK4 or cyclinD/CDK6 causes partial release of E2F activating transcription factors that is sufficient to activate cyclin E and the cdc25a phosphatase. The latter, by promoting removal of inhibitory proteins from CDK2, activates the CDK2/Cyclin E complex that completes the hyperphosphorylation of RB, thereby fully releasing E2Fs. This in turn leads to activation of the latter's target genes and entry into S phase. A second parallel and cooperative pathway also converging on cyclin E/CDK2 activation starts with c-myc proto-oncogene.

Studies carried out on murine ESCs have shown that their cycles lack such sophisticated control [10]. Multiple lines of evidence suggest RB and related proteins are not active in ESCs in which G1 phase is unusually short (c. 1.5 hrs), with most of the cycle being taken up by S phase. Both cyclin E/CDK2 and cyclin A/CDK2 seem to be constitutively active throughout the cell cycle, the only regulators showing obvious cyclicity being Cdk1 and cyclin B1 during the relatively short G2 phase.

ESCs are also atypical in response to DNA damage induced by irradiation, chemicals, or through nucleotide deprivation in arresting in G2 rather than G1, and also in showing serum independence in their cycling. It has been suggested that the short G1 may help to protect the cells against differentiation signals which typically operate during this phase of the cycle in cells which do exhibit a checkpoint.

The G1 phase is also short in human and monkey ESLSCs whose cycles have other features in common with murine ESCs, including resistance to G1 arrest, very low levels of hypophosporylated RB, and constitutive expression of CDK2 complexed with cyclin E but, at least in the rhesus monkey, not with cyclin A. In the case of both ESCs and ESLSCs, exposure to conditions that induce differentiation leads to a very rapid upregulation of expressions of proteins that feature in conventional cycles and a concomitant rise in hypophosphorylated RB.

As noted earlier, key players in maintaining the pluripotent state include Oct4, Nanog, Rex1 and

Sox2. For many years, conditions for derivation, propagations and maintenance of murine ES cells were very ill-defined. Culture was invariably on growth-inactivated feeder cells in either standard serum-containing medium or medium that had previously been conditioned by exposure to other types of cells. Either way, it was supplemented with various growth factors, antioxidants and free-radical scavengers. The two crucial components were held to be leukaemia inhibitory factor (LIF) to promote signalling via STAT3 and either serum or bone morphogenetic protein (BMP) to suppress differentiation. Recent findings have provided compelling evidence that, rather than requiring any external stimulation, sustained proliferation of murine ES cells in an undifferentiated state can be achieved simply by inhibiting key enzymes involved in inducing their differentiation [11]. Thus, use of two small synthetic molecules, one to inhibit the tyrosine kinase FGFR and the other downstream ERK kinases such as MEK, was found to be sufficient to suppress differentiation of ES cells maintained in defined medium under feeder-free conditions [12]. However, in this 'two inhibitor' (2i) medium apoptosis was elevated and the cells showed poor survival at low density. Both limitations were corrected by inclusion of a third synthetic molecule, a specific inhibitor of glucose synthase kinase 3 (GSK 3). Although it remains uncertain why inhibiting this particular enzyme is beneficial, it has been suggested that it relieves negative regulation of biosynthetic pathways. Murine ESC lines that are competent to yield germline chimaeras can now be derived in feeder-free medium containing inhibitors of the kinases FGFR, MEK and GSK3 (= 3i medium) whose only macromolecular additives are transferrin, insulin, and recombinant albumin in place of serum. Moreover, such cultures are maintained in a gas phase with 5% oxygen rather than the unphysiologically high concentration of 20% which had been standard hitherto. Evidence is now emerging that also implicates two distinct families of microRNAs in determining whether murine ESCs differentiate or maintain their stem cell status. These small RNA molecules interfere with gene expression by promoting the degradation of transcripts or inhibiting their translation [13].

A finding of particular value in harnessing ESC transgenesis to provide more tractable physiological models of genetic disease has been the recent demonstration that 3i medium can be adapted to enable germline-competent ESC lines to be obtained in the rat [14], something that had been tried repeatedly in the past without success. However, further underlining the notion that human ESLSCs differ from rodent ESCs is the finding that the former do not survive in 2i/3i medium. Interestingly, this also seems to be true of murine EpiSCs with which human ESLSCs appear to share much else in common.

The exercise of deriving ESCs entails defining conditions that freeze epiblast cells at a stage before they embark on differentiation without compromising their continued proliferation. This has prompted discussion about the nature of such cells for which two contrasting explanations have been offered. One is that their production entails diversion of very early epiblast cells from their normal lineage to yield pluripotent stem cells in which further differentiation has been suspended without impairment of proliferation. The second is that early epiblast cells have simply been arrested at a stage of expansion prior to the onset of differentiation, the initial step in which seems to be conversion of an unpolarized mass of cells into an epithelium. Consistent with the latter possibility is the facility with which ESCs that have undergone prolonged passage in vitro can participate very extensively in normal development following return to the environment of the preimplantation embryo at a stage when it is poised on the threshold of rapid development and differentiation.

The therapeutic potential of ESLSCs

For reasons that are still far from clear, warm-blooded vertebrates show a very limited capacity for regeneration compared with lower members of the phylum. The fault does not necessarily lie with failure of injury or damage to induce proliferation of stem cells but rather the inappropriateness of their subsequent differentiation. Thus, unlike those in the fetus, stem cells induced to cycle in response to trauma in the adult CNS typically go on to differentiate only as glia rather than yield new neurones. Likewise, new growth in response to chemical damage to the liver results in the production of fibroblast type cells rather than new hepatocytes and thus the replacement of functional liver with fibrous tissue. Consequently, one of the major challenges in regenerative medicine is to find appropriate sources of cells or tissue to effect

repair or replacement of damaged parts, and another is to avoid rejection of such grafts.

Regenerative medicine has a long history. It was practiced several thousand years ago in India, for example, to restore noses that had been amputated for various crimes. Here, full thickness skin from elsewhere on the face or from the buttocks was used and, being from the same rather than a different individual, was not susceptible to rejection. Nowadays, taking cells or tissue from elsewhere in the body of patient requiring treatment is practiced widely for such purposes as restoring burnt skin, repairing damaged bone, or bypassing occluded coronary arteries. However, the scope for autografting is limited because there are only certain tissues or organs from which enough material can be obtained to provide a viable graft without further compromising the patient's health. Consequently, cadavers continue to serve as the principal donors of tissues and organs for grafting. Unlike autografts, such allografts do pose problems of rejection except where there is full histocompatibility as, for example, between identical twins.

The first successful allografts were made with corneas from the eyes of cadavers very early in the twentieth century. That these are less prone to rejection than many other types of graft may in part relate to their lack of vascularization. The first enriched stem cell population to be used for grafting was bone marrow which is the primary postnatal source of haematopoietic stem cells. Access to this material obviously depends on an invasive procedure which doubtless deters some potential donors, though effort is being devoted to overcoming this by using cytokines to temporarily mobilize the stem cells from bone marrow to the peripheral blood. The full-term placenta and umbilical cord offer a readily accessible alternative source of such stem cells but, until a way of expanding their number following harvesting can be found, provide only enough material for a single graft. Haematopoietic stem cells are now transplanted routinely in treating patients with leukaemias and other blood disorders, or those who have been subjected to aggressive chemotherapy or radiotherapy for various other cancers.

Use of 'spare' human preimplantation embryos that were surplus to requirements for treating infertility as a source of stem cells for regenerative medicine was, as noted earlier, first advocated by the pioneer of IVF, Robert Edwards. Through their ability to colonize the preimplantation embryo, rodent ESCs have been shown unequivocally to form all types of somatic cells found in the embryo, fetus and adult. This encouraged the expectation that ESLSCs obtained from human preimplantation conceptuses could likewise serve as the source of any type of somatic cell that might be required clinically for tissue repair or replacement. Whether this is really the case remains uncertain because, as noted earlier, analysis of teratomas and differentiated cultures affords a less critical assay for pluripotency than production of primary, or whole-body, chimaeras. Nevertheless, human ESLSCs have been shown to engage in an impressive range of differentiation and, as in the case of cardiomyocytes and hepatocytes, for example, form well organized masses of tissue in vitro. Moreover, rapid progress is being made in defining conditions for optimizing the differentiation of the stem cells along particular pathways.

However, soon after the first human ESLSC lines had been produced claims started to appear in the literature that cells from diverse adult sources were more versatile in their repertoire of differentiation than had been presumed hitherto. Nonetheless, it would be grossly premature to conclude, as some have done, that such findings obviate the need to continue to pursue the ethically more contentious practice of deriving cell lines from preimplantation human embryos. Thus, while several promising sources of adult stem cell have been well characterized, experiments in mice have provided clear evidence that in some cases where cells appear to have undergone major changes in phenotype this resulted from their fusing with cells of the type to which they are supposed to have converted. The resulting polyploidy is potentially hazardous if such fused cells continue to cycle since restoration of euploidy is seldom achieved and aneuploidy is associated with aberrant behaviour, including malignancy. Additional sources of putatively multipotent or even pluripotent stem cells include aborted fetuses and extraembryonic membranes (Table 35.2).

The question of availability of material for allografting in regenerative medicine has thus been addressed by the discovery of a rich diversity of sources from which relevant cells and tissues can be obtained. However, there is also the matter of compatibility of such material with the host requiring a graft. Since, other than between identical twins, the likelihood of complete graft histocompatibility is staggeringly low, recourse to immunosuppression has been

Table 35.2 Source, type and potency of stem cells from different stage of development

Source of stem cell	Type (name) of stem cell	Potency
Epiblast of late blastocyst	Embryonic stem cell (ESC)	Pluripotent*
Epiblast of early postimplantation embryo	Epiblast stem cell (EpiSC)	Pluripotent
Primordial germ cells from extra-embryonic mesoderm and very early genital ridge	Embryonic germ cell (EGC)	Pluripotent*
Various tissues and organ primordial from all three germ layers of the fetus	Fetal stem cells (FSCs) named according to tissue or organ of origin	Multipotent
Extraembryonic tissues and membranes Including both the fluid** and epithelium of the amnion, the placenta, Wharton's jelly and umbilical cord	Named according to membrane or tissue of origin	Multipotent
Spermatogonia from postnatal testis	Spermatogonial stem cells	Pluripotent*
Adult tissues and organs	Named after organ or tissue of origin	Very variable depending on source

*Pluripotent cells shown to be able to contribute extensively to normal development following introduction into the preimplantation embryo.
**Cells in the amniotic fluid include those of uncertain origin within the fetus as well as amniotic origin.

the principal way of avoiding rejection of allografts. Immunosuppressive drugs not only increase the susceptibility of patients to infections and certain cancers, but may also elevate blood pressure and produce other side effects that adversely affect longevity. The birth of Dolly the sheep prompted the suggestion that what became precociously termed 'therapeutic cloning' offered a possible solution to the rejection problem. The proposal was to inject nuclei from healthy cells of a patient requiring a graft into enucleated oocytes which are then activated to develop to blastocysts from which ESLSCs are then derived. Being essentially genetically identical to the patient, these cells could then be used to generate whatever tissue was required for grafting without concern about rejection. This approach is not very realistic in practice for two main reasons. First, the very high rate of anomalous development encountered in reproductive cloning casts doubt on the normality of stem cells produced in this way. Second, the very low efficiency with which embryos have been obtained by cloning means that the necessary supply of oocytes is most unlikely to be forthcoming.

Fortunately, an entirely novel way of obtaining pluripotential stem cells from adult somatic cells has been found recently. This entails transiently expressing in adult somatic cells 3–4 of the key genes that are normally active in ESCs or ESLSCs. This causes a proportion of the cells to revert to a pluripotent state which, in the case of the mouse, has been shown by blastocyst injection experiments to be indistinguishable from that of conventional ESCs [15]. Thus, such induced pluripotential cells (iPSCs) have not only yielded ubiquitous somatic chimaerism but also proved capable of colonizing the germline. So far there seems to be no obvious restriction on the type of somatic cell that can be re-programmed in this way. Further technical refinements are needed before clinical use of iPSCs can be contemplated, but this nevertheless looks to be a most promising way of extending the scope of autografting well beyond what is practical at present. It has also prompted the question whether the ethically more contentious practice of deriving pluripotential cell lines from preimplantation human embryos is still warranted. Obviously, reverting differentiated cells to pluripotency and then inducing their re-differentiation to obtain the desired cell type is rather cumbersome compared to employing pluripotent cells as starting material. However, conversion of postnatal fibroblasts directly to neurones by expressing just three neural genes within them has been demonstrated very recently [16]. It remains to been seen whether such direct interconversion of other types of postnatal somatic cells can be achieved as efficiently and, most importantly, whether impairment of the integrity of the genome in somatic cells is sufficiently common to make those from early embryos a safer option.

The nearest prospect for clinical application is where post-mitotic cells of a specific type are

required, such as dopaminergic neurones for treating Parkinson's disease, or coherent sheets of myocardial tissue for repairing cardiac infarcts. Producing more sophisticated tissue architecture in vitro offers a greater challenge, though initial steps towards this have been taken using appropriate biological matrices or scaffolds that have been divested of cells, proteins and other potentially antigenic components. Autografts for repairing or correcting defective development of the urinary bladder have been produced by seeding cells biopsied from its epithelial and muscular layers on the inner and outer surface of a cup-shaped matrix [17]. This yields a fully cellularized hemi-bladder that can then be ligated into the resident organ *in situ*. Using extracellular material from a cadaver as a scaffold on which the appropriate cells biopsied from a patient can be seeded has proved spectacularly successful recently in restoring function of the left lung whose bronchus had become occluded by a tubercular lesion [18]. Here, the trachea from a dead donor was exposed to multiple cycles of enzymatic treatment to free it of all cells, before its inner surface was seeded with cells biopsied from the patient's airway, and its outer surface with bone marrow-derived mesenchymal stem cells that were incubated in appropriate conditions to induce them to form cartilage. Rotating the trachea in a bioreactor which ensured that its outer surface was colonized by cartilage and its inner one by airways epithelium provided a structure that could be shaped to replace the defective bronchus and thus fully restore pulmonary function. While there is undoubtedly further scope for exploiting cadavers as a source of matrix rather than tissue for achieving more complex arrangements of host cells, producing organs of the complexity of the kidney is still a very remote prospect. Here, consideration needs to be given to using organ primordia from abortuses as an alternative to adult organs [19]. Recent experiments in mice have proved encouraging in showing that the fetal metanephroi can enlarge and function when transplanted into a histocompatible adult [20]. Research is also being undertaken on the development of non-biological scaffolds which may lend further flexibility to the task of organizing more complex tissue architecture in vitro

So far, it has seldom proved possible to obtain in vitro from pluripotent stem cells pure populations of the desired type of cell free from contamination with others whose nature is often uncertain. Rather than being a deficiency in technique, this could reflect the stochastic nature of the differentiation process itself since there is some evidence for the existence in vivo of mechanisms for the elimination of inappropriately differentiated cells. Nonetheless, using contaminated cultures for grafting could obviously prove hazardous so that means of purging them of unwanted cells have to be found. This can be achieved by means of a fluorescence-activated cell sorter (FACS) if antibodies specific to surface components of the desired cell type can be produced without compromising their viability. A very high degree of purity may be achieved by passing cells twice through such a machine. An alternative and rather more complex and controversial approach is to transfect the stem cells with the coding segment of either a gene for a

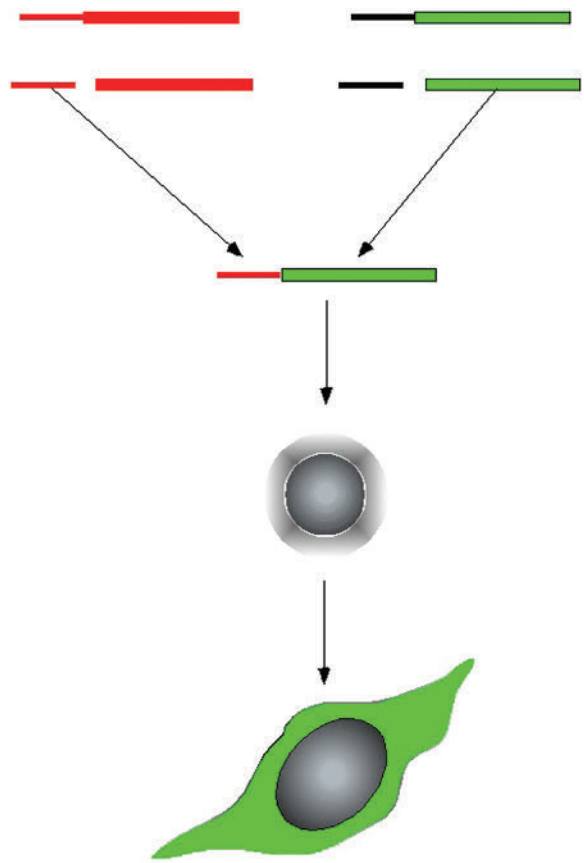

Figure 35.3 Selection of specific differentiated cells by isolating the control region of a gene (shown in red) that is expressed specifically in the desired type of cell and splicing it to the coding region of a gene (shown in green) specifying a protein that is either fluorescent or confers resistance to an antibiotic. Pluripotential stem cells are then transfected with this hybrid gene before being induced to differentiate, and the desired differentiated cells selected by FACSs analysis or exposing the culture to the appropriate antibiotic.

fluorescent protein or an antibiotic-resistant one spliced to the control region of a gene whose expression is specific to the desired type of differentiated cell (Fig. 35.3). Inappropriately differentiated cells can then be removed by means of FACS sorting or killed by exposure to the relevant antibiotic.

A further issue concerns the status of cells required for grafting, namely whether a self-sustaining population of stem cells is required, as would be the case for structures like the skin or intestine that engage in constant turnover, or a strictly post-mitotic population. The former poses the challenge of characterizing the 'niche' that supports the renewal of the relevant stem cell and ensuring that this can either be carried over in the graft or re-created in the host. There is also the further question of whether healthy cells will fare any better that resident host ones when seeded in a damaged organ. Additionally, regardless of the source of stem cells used to provide tissue for grafting, it is vital to show that the latter is normal physiologically rather than, as too often at present, simply in terms of its gene expression profile.

Conclusion

Clearly, knowledge gained about the biology of pluripotential stem cells offers exciting prospects for extending the scope of regenerative medicine. It is important, however, to keep an open mind as to how such cells might be exploited most effectively. It is conceivable, for example, that better understanding of their biology, particularly conditions that dictate the choice of lineage for differentiation, may provide insight into how to unlock endogenous regenerative potential to an extent that might render their use as grafts unnecessary. Moreover, combining host-derived cells with matrices from dead donors offers a way of building complex tissue architecture while avoiding problems of graft compatibility, or the use of non-biological synthetic substrates.

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Chapter 36

Ethical considerations for clinical embryology

Paul R. V. Johnson

Introduction

The field of clinical embryology is advancing year by year, with novel treatments rapidly translating from 'bench to bedside'. In addition, these scientific developments are being made in a highly emotionally charged environment in which the individuals seeking these treatments are often naturally desperate to do anything to be able to conceive. However, as with all areas of clinical medicine, it is vital that such treatments are practised within a carefully determined ethical framework. Indeed, many of the newer treatments frequently challenge the existing frameworks, and it is therefore important that anyone studying or practising clinical embryology does so with a good understanding of the ethical context. The aim of this chapter is to provide an overview of medical ethics as they apply to this field. It starts by defining the scope of medical ethics and clarifying some misunderstandings.

Definitions

While there is a clear overlap between ethics and moral philosophy, the main purpose of medical ethics is to clarify the moral issues, give principles on which to act and guidance in the detail of making right decisions. It is therefore not just the domain of the academic ethicist, but instead is a practical subject in which every member of the clinical multidisciplinary team must engage.

Clarifying the issues

There are a number of myths and misunderstandings that are commonly expressed when medical ethics are being discussed. It is important to clarify these before exploring the practice of medical ethics in more detail. Frequent misunderstandings include:

'Misunderstanding the comparisons'

Before any meaningful ethical discussion or decision-making can occur, it is vital that the key issues are clearly identified and fully understood by all parties. It is very easy for different parties to be discussing with each other at cross-purposes because the issues have been misunderstood or interpreted differently. A good example of this is in the field of end of life care. There are clearly distinct ethical differences between switching off the ventilator of a brain-dead baby on the one hand, and active physician-assisted ending of life on the other. Between these two extremes, however, are more subtle differences between withdrawing and withholding treatment, and between opiate analgesia given with the primary purpose of relieving pain, and the conscious administration of opiates for their respiratory depressant effects. The ethical directives for each scenario may be distinctly different, meaning that all involved with the decision-making process must be fully familiar with the precise facts of the case, and as a result, the precise ethical and moral issues to be considered.

'Medicine can produce its own ethics'

If only this were true! Unfortunately, medical ethics involves applying clearly defined ethical principles and guidelines to a clinical situation, rather than expecting the clinical situation to magically create its own ethical answers. Indeed, medical science is ethically neutral. Fortunately, much of medical practice around the world does have the patient's well-being at its centre and is fundamentally 'good'. However, there are clear examples both in history (and sadly still sometimes in the present) of when medicine has been used in a harmful way without the beneficence of the 'patient'

Textbook of Clinical Embryology, ed. Kevin Coward and Dagan Wells. Published by Cambridge University Press.

as the priority. In addition, there can be times when the absolute aim of some novel treatment is to benefit the patient, and yet that treatment is in itself harmful. An example of this might be a novel potent chemotherapy agent that aims to cure an aggressive tumour, but that during clinical trials is found to have side effects that result in a high mortality rate. While ethics have to be applied to specific clinical situations, it is potentially dangerous to start with the precedent that medicine can form its own ethics. In addition, it is important to emphasize that there is a distinction between applying an ethical framework to a specific situation, as opposed to 'situation ethics' in which one's ethical frameworks are dictated by each different clinical situation. It is true that no two clinical situations are identical. However, 'situation ethics' at its extreme can result in an absence of ethical frameworks and inconsistent ethical decisions being made based on subjective rather than objective criteria.

'There is only one answer to an ethical problem'

This encompasses two interrelated misunderstandings, namely that there are no ethical grey areas, but also the corollary to that, namely that there are no moral absolutes. Neither extreme is true. Occasionally ethical dilemmas are clear-cut with uniform agreement. However, ethical decision-making is seldom straightforward and often involves weighing competing ethical principles, and sometimes involves making a balanced decision based on the principle of the 'lesser of two evils', in which different weight is given to two values, both of which are not ideal. On the other hand, the notion that there can be no 'right' or 'wrong' decision is also misleading. Ethical decisions need to be able to be implemented in practise, rather than persist at the level of theoretical debate. As such, there is often more than one answer to an ethical dilemma, and that is where principles such as 'consensus' and 'precedent' need to be applied.

'Emotion should be kept out of ethical discussions'

It is easy to see how this misunderstanding has been propagated. Clearly, if one allows emotions to completely dictate ethical decision-making, there are huge dangers of ethical fluidity. Taken to its extreme, it can even lead to unethical practice, in which the ethos of 'if it *feels* right, do it' is the dominating driving force. On the other hand, practising medical care solely based on reason and devoid of emotion also clearly has its dangers. Indeed, I would humbly suggest that anyone practising any aspect of medical science in an emotional vacuum should carefully reconsider his or her vocation! Clearly a balance has to be struck. Clinical decisions do engender strong emotional responses, especially in the context of extreme suffering. It must be remembered that at the centre of all medical ethical decisions is a fellow human being with a real clinical problem.

Foundation values

Another misunderstanding that frequently surrounds the field of medical ethics is that the personal beliefs of healthcare professionals should have no effect on their clinical practice. An extension of this misunderstanding is that religion has no part to play in medical ethics. It is really important to explore these misunderstandings further, because in reality, individual and corporate values form a foundation to any ethical decision. Indeed, it is impossible to make any ethical decision devoid of these.

Certainly, individuals have to practise healthcare within existing ethical and legal frameworks. However, every individual brings a preexisting set of values to the decision-making process. This applies equally to whether an individual is an atheist or a follower of one of the principal monotheistic religions (Judaism, Christianity, Islam). At the heart of ethical decision-making are the principles of what is the 'right' decision for this patient, and what is 'good' for them. Different value systems place very different values on questions of 'right' or 'good'. For example, when considering the value of a newborn baby girl with Down's syndrome, the main monotheistic religions would base their value on their view of the child being a human being with a spiritual component to her life. Secular utilitarians would assess this baby's value in terms of her quality of life, and also consider the long-term costs to society as a whole. Some extreme modern Darwinists might consider that the value of this baby is no different from any body organ (some have cited the value of an appendix in their arguments). Based on such a value system, suggesting termination of this baby's life is not an extreme conclusion. Clearly these are extremes, but serve to illustrate that ethical decisions start with values, and values in turn are greatly informed and shaped by one's value system. Also, we must not forget that every one of us operates within a certain value system.

Ethics and law

While medical ethics and medical law are closely interrelated, they are also distinct. Indeed, it is possible to have a situation that is 'legal', and yet might still be 'unethical', and vice versa. Law forms the regulatory framework in which medicine is practised and ethical decisions are made. In the UK it comprises a number of components. Parliament passes certain statutes or Acts on medical issues that dictate what is permitted in medical practice (Statute Law). These can be brought to Parliament by individual members of parliament, or by the government of the day. Frequently these issues have been previously considered by Special Advisory Committees. Examples relevant to the field of Clinical Embryology include the Abortion Law (1967), the Human Fertilisation and Embryology Act (1990) and the Mental Capacity Act (2005). Sometimes, however, Statutes are necessarily ambiguous in their wording, so they can be open to broad interpretation. It can also be difficult to apply them to all situations. The law also makes rulings on individual ethical decisions (Case Law). Such decisions also then create legal precedents against which future ethical dilemmas can be evaluated. The law is also responsible for regulating and licencing healthcare practice, through organizations such as the General Medical Council and the Royal College of Nursing. Such organizations dictate the rules of practise to which healthcare professionals must adhere. The symbiosis between ethics and law is therefore an important one. Clinical ethical decisions help determine some legal rulings, but medical ethical decisions are also bound by existing laws. Indeed, there are times when the law can demand that a doctor breaks certain ethical obligations to his or her patients for the sake of the public good. Examples of this include the requirement under the 1984 Public Health (Control of Diseases) Act for doctors to report certain infectious diseases to the local public health authorities even though this breaks the patient's confidence, and the obligation in certain cases for a psychiatrist to divulge highly confidential information obtained through the doctor-patient relationship if the patient is being tried for a criminal offence.

A framework for ethical guidance

When seeking ethical guidance, it is helpful to have a framework within which to work. Shortly I will address the practical issue of how to come to an ethical decision in practice. However, it is important to emphasize that ethical guidance needs to be tested against some pre-formed criteria. These are termed *Ethical Theories*. These in turn are informed by values to produce *Ethical Principles*, from which *Ethical Rules or Ethical Guidelines* can then be formed (see Fig. 36.1).

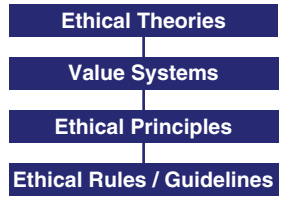

Figure 36.1 A stepwise framework for ethical decision making

Ethical theories

Three main ethical theories form the basis for medical ethics. Their detailed consideration is beyond the scope of this chapter, but for completeness I will include a brief summary of each. In addition, there are different forms of subjective individual ethics that do not rely on ethical theories. These include relativism, postmodernism and existentialism.

Deontology – duty-based ethics

This theory emphasizes obligations on the doctor based upon good, universal, moral principles. It is the theory of moral absolutes, and the branch of deontology termed 'rule deontology' dictates that moral acts are compared against a set of rules or moral principles to determine whether they are 'right' or 'wrong', 'ethical' or 'unethical'. The extreme form of deontology is 'absolutism', in which it is believed that some ethical principles are absolute and cannot be overridden under any circumstances.

Consequentialism (teleology)

Consequentialism, on the other hand, rejects the idea that absolute or universal moral laws can be brought to a decision, but places the emphasis on the consequences of any particular action. The purest form of this ethical theory is 'utilitarianism' with the well-known expression 'the greatest good for the greatest number of people'. This theory places an emphasis on pleasure, and can also involve the principle that the 'end justifies the means'.

Virtue ethics

Virtue ethics rejects the idea of forming binding rules or duties, seeing these as negative and operating

outside the important aspects of relationships. Instead, it concentrates on the attitudes and characters of individuals to do what is 'good' and 'right'. It is true that rules without motivation and compassion can become legalistic. On the other hand, virtues without any framework can easily become subjective and confused.

While the three ethical theories are distinct from each other, in practise one often draws on a combination of these to develop a balanced framework in which to consider ethical dilemmas.

Ethical principles

Five main ethical principles govern medical ethics. As will be seen later in this chapter, although each of these principles is distinct, they are frequently in competition with each other when an ethical dilemma is being evaluated. The following is a summary of each ethical principle.

Autonomy and consent

Autonomy is the right of patients to make decisions about their own treatment, free from the controlling influence from others. Its practical outworking is in the form of Consent to Treatment. Autonomy is being emphasized increasingly in medicine as a response to the previous era of medical paternalism. However, absolute autonomy is not possible, as autonomy has to be practised within a framework in which decisions by one person may affect someone else with an equal right to autonomy. Some would make the qualification that autonomy should be championed, but with the exception of when it causes harm or threatens another person or their property. Consent is a central part of any medical treatment and is bound by the law of 'assault and battery'. This means that any medical practitioner who performs a procedure on a patient without their or their legal guardian's informed consent breaks the law. There are three major requirements for consent to be valid: (1) Consent must be truly 'informed' (i.e. the patient must have the risks of the procedure clearly and honestly explained to them, and in turn they must be able to understand what they have been told. (2) The patient must make the decision voluntarily with no undue pressure placed on them to consent. (3) The patient giving consent must be competent and have the 'capacity' to do so. In most countries there is a legal age at which individuals are legally permitted to consent. In the UK that is at 18 years of age, although individuals under this age can consent to treatment if it is deemed that they are 'Gillick competent' (now termed Fraser competence). The phrase Gillick competence arose from a landmark case in which a mother, Victoria Gillick, brought a case against her local health authority as a result of a physician prescribing the contraceptive pill to her daughter, who was under the age of 16 (the legal age for sexual intercourse in the UK). She lost the case as the child was deemed to have enough 'competence' to be able to understand the issues and to be able to consent to treatment without parental consent. This only applies to consenting for treatment, and specifically does not apply to under-18-year-olds refusing life-saving treatment. It is potentially possible to encounter the situation where a young teenager mother is unable to consent for treatment for herself, but is the legal guardian for her baby!

In addition to age, patients giving consent must be deemed to have the mental capacity to do so. Clearly patients who are unconscious do not have such capacity, and consent is given in those situations by legal guardians with the power of attorney. The same is true for those with severe mental illness. The Mental Capacity Bill (2005) helps clarify the definitions of mental capacity, as well as the code of conduct for gaining consent from those deemed not to have capacity.

Beneficence

Beneficence is the action of 'doing good' for one's patient, and it is central to all medical practice. Non-maleficence is the counterpart of this, and involves doing no harm. However, in practice, difficult decisions have to be made concerning the balance between doing positive good, making sure good outweighs harm, preventing harm, and doing no direct harm. A good example of this is chemotherapy, where the treatment may ultimately be 'good' for a patient, but while causing considerable harm. Also, one's value systems can considerably influence what one considers 'good' for another person. One of the constant decisions that everyone in healthcare has to make is whether a particular treatment or action is 'in the patient's best interests'. Scientific advances can often help us to do good for our patients, but science cannot tell us what is good for them. In the field of clinical embryology, one has to consider the beneficence of the mother, as well as

that of the unborn baby, although legally the mother's well-being takes priority over that of the embryo / fetus. The ethical considerations become more equal once the baby has been born, and so when one is considering the ethical implications for novel treatments for infertility, the beneficence of the child that is ultimately born as a result of these must be brought into the equation. To give an extreme example, if a new treatment enables a mother to conceive and have a longed-for baby, but that treatment inherently causes the baby to live for only a couple of years due to some inevitable genetic alteration, the beneficence of the child would outweigh the beneficence of the mother (beneficence to conceive) when this treatment is being considered ethically.

Confidentiality

Confidentiality is perhaps the most familiar of all the ethical duties in medicine. Indeed, the doctor-patient relationship is centred on trust. Unfortunately, confidentiality is often breached inadvertently. Classic examples of this are when medical staff speak to the relatives of a patient without that patient's prior permission, or when medical staff discuss details of a patient's conditions in hospital corridors or other public areas of the hospital. It must be remembered that for a patient with mental capacity, any medical practitioner should seek the permission of the patient before discussing confidential aspects of treatment with the patient's relatives, or with healthcare professionals not directly involved in that patient's care. Although children are of a different legal status, those dealing with paediatric patients should still respect the confidentiality of the child and their family. This includes restricting access of patient records to those directly treating the patient. Confidentiality is of paramount importance in the field of assisted fertilization, and not only involves the couple being treated, but also the confidentiality of sperm or egg donors if they are involved.

Justice and fairness

In the UK, we often take it for granted that an integral part of the Beveridge Report that formed the blueprint for the National Health Service (NHS) was that there should be 'a health service providing full preventive and curative treatment of every kind to every citizen without exceptions'. Sadly this is not the case in all countries around the world, and the financial constraints of the NHS are also increasingly stretching the ability to deliver these original ideals. There is often now clear variation in treatment availability in different parts of the country, with those areas of social deprivation often most affected. The whole premise for 'justice' and 'fairness' is that people are of equal worth and have equal rights. Clearly with a limited healthcare budget, priorities of healthcare spending are inevitable. However, these should be based on patient need, rather than patient status, and the same rationalization of treatments should be available to all. When offering the range of fertility treatments, clinical embryologists must ensure that justice and fairness are maintained.

Truth and integrity

Truth and integrity are central to the relationship between a healthcare worker and the patient, and the basis for the patient's trust. However, not very long ago it was common practice for some doctors to routinely alter histology reports to ensure that the patient was given a more favourable diagnosis! Interestingly, the driving force for such actions was to protect the patient and it was thought to be 'in the patient's best interests'. Patients, however, have the right to know the truth about their illness, and such behaviour would nowadays correctly result in major sanctions for a doctor in most countries. Healthcare workers need to be truthful about prognosis, and about the true benefits / outcomes of any given treatment. 'Truth' and 'integrity' also need to be paramount in the field of surgical research or the development / testing of novel treatments, where 'conflicts of interest' abound, and the temptation to exaggerate the benefits of a new treatment all too common.

Ethical decisions in practice

One of the important things to appreciate with medical ethical decision-making is that in most difficult ethical situations, the ethical principles described above are competing with each other rather than existing in isolation. Integral to any ethical decision, therefore, is the important step of deciding precisely which ethical principles are involved, and then to determine which principles take priority over the others. For example, in clinical embryology, the autonomy of the mother, the autonomy of the father, the autonomy of the unborn child, and the autonomy of the doctor may

all need to be considered. Clearly, in treatments where multiple embryos are produced, but only small numbers implanted, the ethical principles of autonomy and beneficence may be distinctly different and competing for the mother and the non-implanted embryo. Treatments that are unduly expensive for the healthcare providers may involve difficult ethical considerations of the autonomy of the couple wanting a baby, versus the justice for patients with other conditions also needing to be treated from the same limited budget. The examples of conflicting ethical principles are numerous. It is therefore vital that a clear ethical pathway is formed in order to address a particular ethical scenario and come to an ethical decision. The pathway includes the following important questions:

What value system undergirds the ethics?

We have already seen that different value systems influence ethical decisions. There must be clarity from the outset, therefore, as to what value systems are being followed by each individual involved in the decision-making process.

Is the overall aim of the treatment being considered ethical?

Usually this is clear, but sometimes a particular therapy may in itself be unethical from the outset.

Are there any myths or misunderstandings?

This enables the precise issues involved to be clearly defined, and ensures that all parties involved are in agreement as to what the issues are.

Who and what are involved?

Many ethical problems involve many different groups of people. As with the myths and misunderstandings, these need to be clearly defined from the outset.

Which ethical principles are involved?

All the ethical principles that are involved with the particular ethical problem need to be identified so that they can be included in the ethical evaluation. Often more principles are involved in a particular ethical situation than initially thought.

Are any of these in competition with each other?

As outlined above, they frequently are, and identifying this clearly is central to any ethical decision.

Can the competition be resolved by changing the method or technique of the treatment?

In the field of assisted conception, an example would be to restrict IVF and embryo transfer to one embryo, in which the ethical dilemma of storage and disposal of multiple embryos ceases to be an issue. Often, however, it is not possible to modify the treatment.

Should one ethical principle take priority?

Occasionally it is clear that one ethical principle takes absolute priority over the others. However, frequently we are dealing with relative priorities, or the situation of 'lesser of two evils'.

What are the likely long-term consequences that an ethical decision will have?

Those considering the ethical problem need to evaluate whether the proposed treatment (or clinical study) will result in predictable side effects or long-term consequences that can be predicted, and to determine whether these are acceptable or not.

Is there already professional guidance about this ethical problem?

In the field of clinical embryology, the Human Fertilisation and Embryology Authority (HFEA) has produced clear guidance on different aspects of treatment.

Is there already legal guidance about this ethical problem?

As outlined in the section above on 'Ethics and law', sometimes clear legal rulings / guidelines exist for a particular ethical problem.

Does this ethical problem need to be referred to a research or clinical ethics committee?

All clinical research projects involving human subjects need to be submitted to an ethics committee for approval before the study can be conducted. Also, nowadays, all new operations or procedures being introduced to a particular hospital (even if that procedure is being performed elsewhere) need to be evaluated by that hospital's committee responsible for 'New Treatments and Innovations'.

Will a particular decision start a precedent?

Any ethical decision has the potential to provide a precedent for future ethical problems. This needs to

be taken into account when any new decision is being made, and all decisions need to be taken responsibly.

Is the rest of the healthcare team in agreement with the ethical decision?

Clearly there are times when different members of the multidisciplinary healthcare team (or the patients themselves) come to different conclusions when considering an ethical dilemma. However, in order to be able to implement an ethical decision for a particular clinical scenario, consensus has to be reached. 'Opt out' or 'conscience clauses' exist for healthcare workers who feel that their personal beliefs do not permit them to participate in particular treatments. While the tendency is for these to be eroded, it is imperative that these clauses be maintained, so that the autonomy and values of healthcare workers are respected.

Once the decision has been reached, has this been communicated to the patient?

Surprisingly this important aspect is frequently delayed and sometimes even forgotten. It is important to remember at all times that the patient is at the centre of the ethical decision-making process.

Conclusions

The field of clinical embryology requires all who are involved in it to be able to think and practice ethically. The field is changing rapidly, and ethical frameworks are becoming challenged with ethical dilemmas of ever-increasing complexity. The aim of this chapter has been to provide the background, framework and basic principles of how to address specific ethical dilemmas, and to outline how an ethical decision can be made in clinical practice. The reader is now encouraged to study the ethical aspects of specific treatments in more detail. For further discussion regarding the ethical and legal implications of ART, please refer to Chapter 20.

Further reading

A. G. Johnson and P. R. V. Johnson. *Making sense of medical ethics – a hands on guide*. (London: Hodder Arnold, 2007).

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