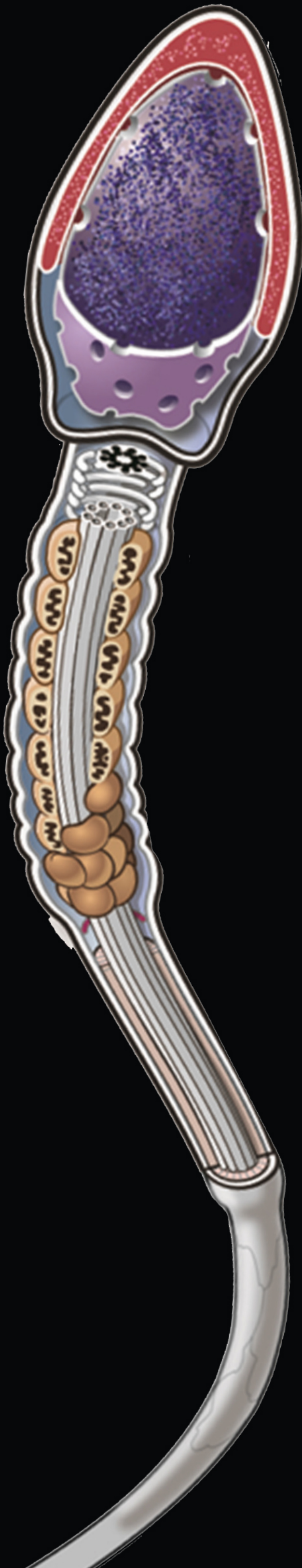
A PRACTICAL GUIDE TO SPERM ANALYSIS

Basic Andrology in Reproductive Medicine



Edited by
Nicolás Garrido
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A Practical Guide to Sperm Analysis

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Basic Andrology and Reproductive Medicine

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Preface

Assisted reproductive technology is a rapidly evolving field where new information about diagnostic and therapeutic options is constantly arising, making all specialists need assistance in the correct interpretation of each aspect in order to provide the best care for their patients, gametes, or embryos.

In this sense, the male factor has been historically neglected in a specialty led mainly by gynecologists and focused on the female. The male's contribution may account for a significant influence on reproductive success, starting from the almost 50% of the embryo's composition (if one considers that maternal mitochondrial DNA is conserved). But from the therapeutic viewpoint, this contribution may be even higher, considering that the sperm to be employed in the assisted reproduction treatment can be selected among millions, and this selection may decide reproductive success or failure, when all oocytes available are employed.

Sperm quality measurement has been classically done under the parameters established by the World Health Organization manuals. This non-evidence based approach, instead of clarifying things, led to confusion among patients, practitioners, and lab specialists, due to the lack of predictive power in natural conceptions, and also the lack of linking with assisted reproduction results.

Since intracytoplasmic sperm injection (ICSI) was introduced, there has been a significant decrease in the interest to study sperm physiology and function, assuming that all the work needed from the male side was already done mechanically from the IVF center's operators. Now, decades later, we know that ICSI may overcome fertilization problems but not ulterior physiological events, where sperm physiology still plays a role until the embryo takes the responsibility. Also, the genetic issues related to poor sperm quality have joined the clinical scenario, once ICSI children were evaluated, opening a different area of concern from using these techniques.

More recently, the availability of molecular biology techniques able to analyze hundreds or even thousands of molecules has opened a new approach to male fertility and sperm fertility evaluation.

Now that there is enough evidence to be sure that sperm function is multifactorial, many different molecular markers have been suggested as being involved in sperm-correct physiology. Even more importantly, different cellular biology techniques, either those coming from other biomedical specialties or those specifically developed for sperm, that permit the isolation of single spermatozoa, while keeping their integrity and viability on the basis of molecular traits, are being tested in order to be implemented in assisted-reproduction laboratories to enhance a couple's reproductive chances.

This leads us to the historical doubt about treating the male or the sample.

As more and more information is available, the interpretation becomes more difficult, hence the need for specialists to describe the biological basis, techniques, interpretation of the results, and the reproductive counseling afterward, in order to assist our patients. This will expand the link between science and clinical practice, permitting the translation of scientific knowledge into practice.

We are very thankful to all contributors of this book, world opinion leaders on their topics and corresponding areas, for their speedy and fluent contribution, as well as for the quality of their work, which will probably (and hopefully) make this book a reference in the field.

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1

The Usefulness of Sperm Viability Testing in Reproductive Technology: The Hypo-Osmotic Swelling Test, Laser and Motility Stimulants

Denny Sakkas

The Scientific and Biological Bases of Testing Sperm Viability

It is commonly accepted that the standard World Health Organization (WHO) criteria for sperm number, motility, and morphology are a good, although not always perfect, indication of a male's fertility status. The current criteria[1] have recently been changed from the 1999 to 2010 WHO recommendations, whereby semen volume (2–1.5 mL), sperm concentration (20–15 million per mL), progressive motility (505%–32%), and normal forms (14%–4%) have all been decreased, respectively. Although the current criteria suggest a certain volume, number, motility, and morphology, it must be emphasized that this is an indication that the male may have difficulties to father a child rather than predicting fertility.

Fortunately, the majority of these males that fall below these semen parameters will conceive even though it may take longer or they will need assistance through one of the assisted reproductive technology methods. Some, however, will be diagnosed with extremely low or absolute asthenozoospermia, and even with the assistance of intracytoplasmic sperm injection (ICSI) it becomes difficult to treat this condition.

These extreme cases of ICSI where there are no motile spermatozoa, extremely few motile spermatozoa, only twitching spermatozoa, or when sperm numbers are extremely low now represent the most challenging treatment situations. The advent of no motile spermatozoa may arise due to a number of scenarios not excluding immotile cilia syndrome, which is thought to be present in approximately 1 in 5000–6000 men.[2,3] More frequently, the presence of no motile spermatozoa will arise after sperm are extracted from the testes or epididymis or after a low-quality sample is frozen and thawed.

Along with the presence of no motile spermatozoa is also the inability to distinguish if those spermatozoa are in fact viable or dead. It is well accepted that the arbitrary selection of an immotile spermatozoon and attempt at fertilization by ICSI will provide significantly lower chances of success.[4–8] The challenge, however, is how to treat these men, indeed the question is, "When an ICSI technician is deprived of the chance to choose a motile spermatozoa how does he/she find a live sperm?" This chapter discusses the various options that allow in vitro fertilization (IVF) clinics to improve the chances of success for couples in which the male has extremely poor sperm parameters that limit the chance of selection of a viable sperm.

Analytical Techniques to Test Sperm Viability

The standard and recommended viability test for sperm is that recommended by the WHO manual. The test in effect identifies which sperm have an intact membrane by either excluding a particular dye or hypotonic swelling. Briefly, dye exclusion means that any damaged plasma membrane will allow entry of membrane impermeant stains; it entails a vitality test using eosin–nigrosin where live spermatozoa will have white heads and dead spermatozoa will be red. Eosin alone[1] is also an option for testing viability. Some commercially available options are provided, for example, Sperm VitalStain from Nidacon, Sweden.

Unfortunately, the use of any stain precludes an individual sperm from being used clinically. Hence, the act of staining, although providing information about viability, will not be useful. Another test that is commonly used to assess viability is the hypo-osmotic swelling test (HOST), which acts because sperm with intact membranes are not leaky and will swell as they are able to retain fluid leading to coiling of the tail. The test was first described by Jeyendran et al.[9] in 1984 and is a good indicator of the functional integrity of the sperm membrane.[6] Its use in being able to select viable nonmotile sperm is popular because of the simplicity of the test. One problem with the test is that it may be less accurate when frozen–thawed spermatozoa are assessed as they experience a higher rate of spontaneously developed tail swellings and that this can exaggerate the HOST score.[10]

Clinical Options with Nonmotile Spermatozoa

As seen in the earlier section, nonmotile spermatozoa do not necessarily mean dead spermatozoa, hence when applying ICSI to these cases it is virtually imperative that this distinction be made. The term extreme ICSI was coined recently in an article by Palermo et al.[11] The extreme in this article refers more to treatment of men with severely compromised spermatogenesis, including those with virtual azoospermia or nonobstructive azoospermia requiring an extreme search for spermatozoa. In their study they came to the conclusion that in testicular sperm extraction (TESE) patients there was a decrease in pregnancy rate (44%–23%) with increasing time of search for sperm prior to ICSI. More importantly they concluded that, even with extreme ICSI, there is a preference to use spermatozoa that display motility characteristics as even if motility is poor—or there is twitching—it still displays proof of cell viability. Hence, there is a preference in the clinical setting to always use motile sperm, regardless of the quality of motility.

There are, therefore, a number of choices when faced with the prospect of no motile sperm in a sample to be used for ICSI.

1. Select sperm randomly from the nonmotile population.
2. Select sperm using a test that can indicate viability without damaging the sperm, for example, HOST or laser-assisted selection.
3. Stimulate the nonmotile sperm so that it achieves some motility.

Clinical Implications of Using Immotile Sperm for ICSI

No large specific data sets exist on the use of totally immotile spermatozoa for ICSI as in many cases an attempt is always made to select some motile spermatozoa; some cases proceed with a mix of embryos fertilized by both motile (twitching) and immotile spermatozoa. The data, however, are conclusive that the inability to identify motility in a sperm prior to ICSI is detrimental. In an initial study by Nijs et al.,[12] they found that both initially immotile and totally immotile spermatozoa had the capacity to fertilize an oocyte after ICSI, whatever their origin, testicular or epididymal. Totally immotile ejaculated spermatozoa fertilized significantly fewer oocytes after ICSI when compared with initially immotile ejaculated spermatozoa. Embryos of lower quality tended to be produced when totally immotile spermatozoa of any origin were used compared with embryos resulting from initially immotile spermatozoa. Pregnancy rates were also severely reduced when totally immotile sperm were used from the epididymis and ejaculate. Another early study also showed similar tendencies. The microinjection of completely immotile spermatozoa in 11 couples who underwent an initial ICSI cycle with 100% immotile freshly ejaculated spermatozoa resulted in pronuclear fertilization in only a total of 18/145 (12.4%) injected oocytes.[13] None of these cycles resulted in a pregnancy. Although the earlier studies showed that fertilization was not an ultimate impediment when using immotile spermatozoa, a study by Liu et al.[5] has shown that one of the major factors influencing fertilization failure after ICSI was the presence of only immotile sperm.

Clinical Implications of Selecting Viable Immotile Sperm for Intracytoplasmic Sperm Injection

A number of methods have been adopted to select immotile sperm using a test that can indicate viability without damaging the sperm. The most widely and traditionally used test is HOST, which acts as a surrogate measure of sperm membrane integrity or viability. The HOST has been used historically in cases of sperm samples with 100% immotile cells, including those from patients with Kartagener's syndrome.[9,14,15]

Recently, it was also reported that the HOST can identify individual spermatozoa with minimal DNA fragmentation,[16] and with traits of apoptosis, abnormal head morphology, nuclear immaturity, or membrane damage.[17,18] When used for immotile sperm the results indicate that HOST is beneficial for testicular sperm; however, when applied to ejaculated sperm the results are less convincing. Sallam et al.[19] performed a randomized controlled trial in a total of 79 couples with immotile testicular spermatozoa treated with ICSI and examined HOS. In the first group, spermatozoa used for injection were selected using the modified HOST, whereas in the second group spermatozoa were selected based on their morphology. The fertilization rate was significantly higher in the HOST group (43.6%) compared with the no-HOST group (28.2%), whereas the pregnancy and ongoing pregnancy rates were also higher in the HOST group (27.3% vs. 20.5%) compared with the no-HOST group (5.7% vs. 2.9%).

Interestingly, when used on routine ICSI patients the clinical results may support the need to validate this technique prospectively on patients other than those with immotile spermatozoa. Several clinical studies report that the use of HOST-selected sperm for microinjection has been beneficial for implantation and pregnancy rates in patients with immotile sperm[20] and also in recurrent miscarriage couples.[21–23] For testicular sperm, fertilization rates have been shown to be better, yielding increased pregnancy rates in prospective and randomized trials.[19,24] Some concerns have been raised about the use of HOS for ICSI because of a longer exposure to the hypo-osmotic conditions. Barros et al.[25] have proposed some modifications of the classic HOS technique with pregnancies reported.

In addition to selecting viable immotile sperm by HOS, other methods have also been tested. Two methods that rely on a similar strategy to HOS are mechanical touch and laser-assisted selection. The mechanical touch technique was described by Soares et al.[26] and de Oliveira et al.[27] and basically involves pressing against the upper third of the immotile spermatozoon tail and the ICSI dish with the ICSI micropipette forcing the tail to one side. The micropipette is then raised, and the tail response is observed. If the tail is flexible and recovers its original position, the sperm is considered viable. Sperm rigidity and incapacity to recover the initial tail position is considered a sign of nonviability. A number of pregnancies were reported using this technique.

The availability of laser technology in human IVF in the early 1990s[28,29] paved the way for facilitating assisted hatching and in particular embryo biopsy. A number of other applications have also been developed for laser technologies including some that assist in selecting viable immotile spermatozoa. In 2000, Montag et al.[30] reported the use of laser for immobilization of human spermatozoa prior to ICSI. They observed that spermatozoa responded to laser immobilization similar to the HOST, with curling of the tail. In collaboration with a Turkish group[31] they performed the HOST and laser-assisted sperm selection in 10 patients with immotile spermatozoa and found that the mean percentage of spermatozoa found viable was not significantly different (HOS median 21.5% vs. laser reaction median 22.0%). When patients with immotile testicular biopsy sperm were treated they found that laser selection gave higher fertilization and embryo cleavage results when compared with a random selection of sperm. The take-home baby rate was also higher with 4/21 (19%) in the laser sperm selection group versus 1/17 (5.9%) in the random sperm selection group.

Clinical Implications of Making Immotile Sperm Motile for Intracytoplasmic Sperm Injection

Sperm motility has fascinated reproductive biologists for centuries. In the 1950s and 1960s, several researchers used cine- or single time-exposure photomicrography to investigate the characteristics of spermatozoa; however, they were very time-consuming procedures (reviewed by Mortimer[32]). Within the context of

these studies, many researchers initiated investigation into chemicals and their pathways that would affect sperm motility. Some of the most commonly used stimulants were caffeine, pentoxifylline, theophylline, and 2-deoxyadenosine (reviewed by Lanzafame et al.[33]). It was, therefore, a natural progression to investigate whether certain chemicals could reactivate the motility of apparent immotile spermatozoa.

Pentoxifylline and Theophylline

Pentoxifylline and theophylline are both methylxanthine derivatives and act to induce sperm motility by inhibiting phosphodiesterase activity that leads to an increase in intracellular cyclic adenine mononucleotide phosphate (cAMP) levels. Two initial papers from the same group by de Turner[34] and Aparicio et al.[35] reported that pentoxifylline could alter the motility patterns of human spermatozoa. Several years later Tasdemir et al.[36] used pentoxifylline to initiate motility in testicular spermatozoa, suggesting it as a tool for differentiating live and dead sperm cells during ICSI. In their study, 10 immotile testicular sperm samples were divided into two parts for examination of sperm motility with and without pentoxifylline treatment at 30, 60, and 90 minutes. The samples without pentoxifylline remained immotile even after 90 minutes of incubation, whereas the addition of pentoxifylline initiated sperm motility in all samples: 51.8 ± 10.2%, 64.4 ± 9.4%, and 70.8 ± 8.9% (mean ± SD) at 30, 60, and 90 minutes, respectively. Some concerns exist, however, as to the safety of these chemical substances. For example, Scott and Smith[37] found that pentoxifylline, caffeine, 2-deoxyadenosine, and cAMP had adverse effects on mouse oocytes or embryos at concentrations commonly used to activate sperm in human IVF. They concluded that care should be taken to minimize the exposure of human oocytes and embryos to these agents until their direct effects have been investigated more fully. The exposure times to the oocyte are, however, much less than what was adopted in this manuscript. In general, sperm are exposed to approximately 1.8-mM pentoxifylline for 10–120 minutes; the motile sperm are then identified and placed in a wash drop to further dilute the pentoxifylline. Therefore, the amount of chemical finally reaching the egg is minimal. From the clinical pregnancies, reported there has been no identified harm in using this technique. Concentrations ranging from 1.76 to up to 5 mM of pentoxifylline have been reported for use in ICSI cases.[38] In our own laboratory, we use a 3.6-mM exposure and expect the motility of the spermatozoa to be activated within 10 minutes in most cases. If motility is not observed within 20 minutes, we then proceed to use the HOST.

Clinical utilization of pentoxifylline has now become quite broad and pregnancy results have varied in studies from no improvement to significant improvement. For example, Kovacic et al.[39] reported a retrospective study of ICSI performed with immotile sperm without pentoxifylline compared with sperm treated with pentoxifylline before injection. Most cases of totally immotile sperm were seen in thawed testicular samples, but in 27 of 29 samples motility was later triggered with pentoxifylline. They found a significantly higher fertilization rate (50.9% vs. 66%; $p < 0.005$) and higher mean number of embryos per cycle in the pentoxifylline group (2.7 $\pm$ 2.1 vs. 4.7 $\pm$ 3.3; $p < 0.01$). There was no statistically significant difference in the clinical (26.7% vs. 38.3%) and ongoing pregnancy rates per cycle (26.7% vs. 31.9%) between the nonpentoxifylline and pentoxifylline group. Interestingly, the availability of more embryos could mean that more pregnancies could be achieved through frozen embryo transfers. Another parameter that showed improvement was the mean time required for identification and isolation of the sperm and ICSI, which was significantly lower for the pentoxifylline group. The improvement in search time becomes more relevant given the publication in relation to extreme ICSI whereby the trend indicated that pregnancy rate decreased as search time increased.[11] In another study, Ben Rhouma et al.[40] treated immotile spermatozoa for 10 minutes with 3.6 mM of pentoxifylline prior to ICSI. Motility was initiated in all 61 cases. When they compared the results of 72 cycles with motile frozen–thawed testicular sperm (control group) and 61 cycles with immotile frozen–thawed testicular sperm treated with pentoxifylline they found no difference in fertilization rates. From a total of 66 transfers in the control group and 54 transfers in the pentoxifylline group, they had a clinical pregnancy rate per cycle of 36% and 33%, respectively, and a delivery rate of 29% and 28%, respectively.

Although most studies have compared pentoxifylline with nontreated sample, one study by Mangoli et al.[41] compared selection after exposure of immotile testicular sperm to pentoxifylline with the HOST. They reported that even though viable spermatozoa were obtained in both study groups, significantly

higher fertilization rates (pentoxifylline 62.05% vs. HOS 41.07%) and clinical pregnancy rates (pentoxifylline 32% vs. HOS 16%) were observed.

Theophylline is a similar chemical compound to pentoxifylline and has also been used. Ebner et al.[42] performed a study on 65 patients in which sibling oocytes were split into a study using ICSI with thawed testicular sperm treated with theophylline and a control group using ICSI only with thawed untreated sperm. All patients but one (98.5%) showed a significant improvement in testicular sperm motility when theophylline was used. Similar to the studies using pentoxifylline, sperm selection took significantly less time in the study group and rates of fertilization (79.9% vs. 63.3%) and blastulation (63.9% vs. 46.8%) were significantly increased. Significantly more patients achieved clinical pregnancy from the treated oocytes that had been injected with theophylline-selected testicular spermatozoa (53.9% vs. 23.8%).

Although a number of studies have shown that the chemical stimulants pentoxifylline and theophylline failed to improve pregnancy rates[39,40,43,44] some, such as the Mangoli et al.[41] and Ebner[42], have shown benefits in pregnancy. The majority of studies do consistently show that fertilization, embryo number, and the time to recover a motile sperm are significantly improved when using a chemical stimulant of motility such as pentoxifylline or theophylline (reviewed by Rubino et al.[45] and Nordhoff[46]).

Kartagener's Syndrome

Kartagener's syndrome belongs to a heterogeneous group of inherited autosomal recessive diseases, characterized by dramatically reduced or complete absence of ciliar motility.[47] Men affected by this genetic condition may present with various respiratory issues and male infertility. The prevalence can range from 1 in 20 to 40,000 individuals.[48] The treatment of Kartagener's syndrome has been highly successful with ICSI alone[47] and also with increased sperm selections using either HOS or pentoxifylline.[49,50]

A recent review by Davila Garza and Patrizio[51] examined the numerous reports of pregnancies after treatment of Kartagener's syndrome and found that a better fertilization rate was achieved with testicular sperm (65% vs. 55%) compared with ejaculated sperm. They also found that the pregnancy rates were slightly higher in the ejaculated sperm group (45% vs. 35%). The overall live-birth rate was 39% (11 of 28 embryo transfers). A total of 18 babies were reported born and referred to as healthy, with no apparent transmission of this condition to the offspring. The number of offspring worldwide must be much higher as our own clinic, similar to other clinics, has successfully treated these patients (unpublished results).

Clinical Availability of Current Tests and the Future

The future does offer some other interesting new options for selection of viable sperm, in particular, the possible imaging of mitochondrial function using Raman[52] or its use to identify sperm DNA damage.[53–56] Spectra from different regions of the sperm have been described, including DNA within the sperm head, based mainly on changes in a peak at 1092 cm^{-1} (suggested to be the DNA backbone). The advantage of this technology is that the nuclear DNA status can be checked but whether it can distinguish between dead and live sperm is not yet clear.

The tests mentioned earlier are relatively simple given the gamut of technologies that are now applied by IVF laboratories. The more widespread adoption of preimplantation genetic screening has seen a higher use of lasers; therefore, the laser technique of selection may be applied more in the future. The ability to perform HOST or stimulate motility with chemicals such as pentoxifylline is also relatively simple and allows the above-mentioned treatment options to be easily adopted internationally.

The ability to select a viable immotile sperm provides couples where the male has complete lack of motile sperm a valid option to create their own family. It does appear that the use of testicular harvested immotile sperm in these cases does improve their chances. A final consideration when consulting these couples is always to remind them that the cause of sperm immobility can be linked to genetic causes and it is likely that in some cases their male offspring will have the same issue. This of course will raise the ethical conundrum of whether the couple should also consider selecting for a female offspring. These issues will become more complicated for the couple and treating clinician in the future, therefore appropriate counseling will be paramount.

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2

History and Physical Examination: Male Infertility

Jared L. Moss, Mary Kate Keeter, and Robert E. Brannigan

Introduction

Approximately 15% of couples are unable to conceive after 1 year of unprotected intercourse.[1] Research has demonstrated that male factors affect couples' infertility in up to 50% of cases, and a pure male factor is present approximately 20% of the time.[1,2] The first step in diagnosing and treating infertility is an initial evaluation that includes the collection of medical history, a physical examination, and at least one semen analysis.[3] This comprehensive evaluation can provide clues to the etiology of infertility and may detect the underlying disease. According to the American Urological Association's (AUA) *Best Practice Statement on Optimal Evaluation of the Infertile Male*, "Evaluation is warranted in couples who have had unprotected sex for one year without success, or sooner than one year if male or female infertility risk factors exist (including advanced maternal age [over 35 years of age]), or if the couple questions the fertility status of the male partner."[4]

Male factor infertility is typically characterized by at least one abnormality on semen analysis. A full evaluation by a urologist or other specialist in male reproduction is important because many causes of infertility may not be apparent on a standard semen analysis.[4] The most common causes of male infertility are varicocele, idiopathic, obstruction, cryptorchidism, immunologic, ejaculatory dysfunction, testicular failure, drug induced, and endocrine.[5] Screening for genetic causes of infertility should be done for certain patients because results may assist in treatment selection as well as provide insight into the potential impact on future offspring. Infertility may also be an indication of a more severe health condition. For example, some men with cancers such as lymphoma or testicular cancer initially present with infertility, which underscores the need for proper physical examination and testing.[6]

An initial evaluation will help to identify whether the cause of infertility is reversible or irreversible. Most causes including hypogonadotropic hypogonadism, varicocele, and ductal obstruction have the potential to be reversed.[7] Examples of irreversible causes are hypergonadotrophic hypogonadism or primary testicular failure, genetic abnormalities like Klinefelter syndrome and Y-chromosome microdeletion, and anatomical causes such as congenital bilateral absence of the vas deferens (CBAVD). Fortunately, not all irreversible causes will prevent a couple from having a biological child of their own. Many irreversible male factors have been mitigated following the development of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

A complete history and physical evaluation will guide the clinician in determining proper treatment, counseling, and reproductive options for the couple. This chapter provides a review of optimal history and examination procedures for evaluating the infertile male.

History

Male Sexual and Reproductive History

Gathering information about the patient's sexual and reproductive history is essential to evaluate possible infertility. The first step in obtaining a proper reproductive and sexual history is to determine how long the couple has been attempting to conceive. Long durations of infertility may suggest involvement of a more severe male or female factor. It is crucial to inquire whether the patient has

previously initiated a pregnancy with another partner as prior proven fertility suggests that significant derangement in sperm production is unlikely.

Erectile dysfunction and abnormal ejaculation can also negatively impact fertility potential. Many men may be unaware of this association or may be unwilling to discuss these symptoms. The examining physician must elicit this information, especially for men with systemic illnesses such as poorly controlled diabetes mellitus, multiple sclerosis, and Parkinson's disease. These illnesses can cause neurological damage resulting in erectile dysfunction and/or ejaculatory disorders. Additionally, many men with spinal cord injuries will have varying degrees of erectile dysfunction and ejaculatory disorders. They typically exhibit low-volume ejaculate, retrograde ejaculation, or aspermia, which are often reported by the patient as "dry ejaculation" or low-volume ejaculate.

Patients should also be assessed for known risk factors associated with decreased fertility, including but not limited to, recurrent urinary tract infections (UTIs), prostatitis, epididymo-orchitis, postpubertal mumps, and sexually transmitted diseases. Infections involving the genitourinary tract in men may result in obstruction. Obstruction can occur at the level of an ejaculatory duct, vas deferens, or epididymis. Ejaculatory duct obstruction may present with an insidious sign such as isolated low-volume ejaculate.

Developmental History

Special attention should be paid to whether the patient's testicles descended properly as a child. The presence or history of an undescended testicle has been linked to reduced fertility, with approximately 10% of infertile men having a history of cryptorchidism and subsequent orchiopexy.[8,9] Infertility is two times more common in men with a history of unilateral cryptorchidism and six times more common in men with a history of bilateral cryptorchidism.[10,11] The reduction in fertility potential has been attributed to a limited number of germ cells as well as defective prepubertal germ cell maturation associated with the abnormal position of the testicle.[12] Additional data indicate little fertility potential for testicles that are not descended properly in the scrotum prior to puberty.[13] The AUA recommends that orchiopexy be considered in patients who have not had descent of the testicle by 6 months of age because the deleterious effects on the testicle worsen with time.[14] It should be noted that although scrotal relocation of the testis may reduce the likelihood of infertility, it will not prevent it entirely.[9,14]

Absent or delayed puberty is also associated with infertility. Men with these conditions may have an endocrine abnormality such as hypergonadotropic or hypogonadotropic hypogonadism. Klinefelter syndrome, a form of hypergonadotropic hypogonadism, is commonly diagnosed following a developmental delay. Men with Klinefelter syndrome appear to have a decline in the functional capacity of the testicle, and most males become hypogonadal. Histological studies have demonstrated a gradual deterioration of the testes over time with hyperplasia of poorly functioning Leydig cells.[15] Although most males with Klinefelter syndrome are azoospermic, approximately 50%–60% of these individuals will have sperm found in their testicles as adults when undergoing microsurgical testicular sperm extraction (m-TESE).[16] The timing of this procedure remains controversial as there is some evidence that m-TESE may be more effective in the adolescent population prior to the decline of testicular function, but this concept has not been conclusively proven.[17]

Kallmann syndrome is a type of hypogonadotropic hypogonadism. It typically presents as absent or delayed puberty and the prevalence is approximately 1:8000 males.[18,19] The most common phenotypic manifestation of the disease is anosmia and hypogonadotropic hypogonadism. The most prevalent mechanism resulting in Kallmann syndrome is failure of the neurons responsible for secreting gonadotropin-releasing hormones (GnRHs) that migrate into the hypothalamus.[20] These men normally respond to gonadotropic hormonal treatment to stimulate sperm and testosterone production.

Systemic Illness

Certain systemic illnesses can decrease fertility potential in a variety of ways. For example, men with end-stage renal disease are known to have decreased fertility, while conditions unrelated to the genitourinary tract may also significantly impact fertility. As discussed in the section "Male Sexual and Reproductive

History," men with poorly controlled diabetes may have trouble with erections and/or ejaculation, and a febrile illness can impair sperm production for up to 3 months.[21,22] In addition, men with frequent upper respiratory infections and situs inversus might have immotile cilia syndrome, or Kartagener's syndrome, which results in immotile sperm. Men with a history of severe headaches, galactorrhea, and impaired visual fields may have a prolactinoma and are at risk for suppression of gonadotropic hormones (i.e., hypogonadotropic hypogonadism). Prolactinomas are generally detected by checking prolactin levels and pituitary imaging. They are typically treated with medical or surgical therapy. Fortunately, this is typically a benign process and men usually have restoration of their hypothalamic–pituitary–gonadal (HPG) axis and return of fertility following treatment.

Cancer is a known risk factor for male infertility. Prior to initiating treatment, men with testicular cancer and lymphoma have low sperm concentration approximately 60% of the time.[23–25] Ragni et al.[26] reported that over 10% of cancer patients banking sperm at their institution were azoospermic prior to cancer treatment. The exact cause of suboptimal semen parameters in men with cancer is not entirely understood and is likely multifactorial. It has been hypothesized that local paracrine disturbances may exert deleterious effects on the testicles. These effects may be more apparent in patients with lymphoma or testicular cancer due to direct involvement of the testicle. Other studies postulate that systemic endocrine abnormalities may negatively impact sperm production.[27]

Men with cystic fibrosis and those who are carriers of the disease will have CBAVD. This is usually detected during physical examination with absence of the vas deferens and only the proximal third of the epididymis being present. Skip lesions are possible in the vas deferens and may help to account for men who have a unilateral palpable vas and azoospermia. The testicles are generally normal in size, with most men presenting with obstruction rather than a production issue. Genetic screening for cystic fibrosis transmembrane (CFTR) conductance regulator mutations is recommended for men diagnosed with CBAVD on physical examinaion.[4] Sperm extraction is possible, and genetic counseling is encouraged for both the male and female partner, especially if the patient plans to pursue prenatal diagnosis.[28]

Past Surgical History

Various surgical procedures can negatively affect male fertility. The examining physician should inquire about a patient's past surgeries even if the surgery was unrelated to the genitourinary tract. For example, men who have had brain surgery or brain radiation have an increased risk for HPG dysfunction. Common intraabdominal inguinal surgeries such as hernia repair may pose risk to fertility due to damage or obstruction of the vas deferens. Patients with testicular cancer undergoing retroperitoneal lymph node dissection (RPLND) are at risk for damage to the sympathetic nerve plexus resulting in failure of seminal emission or retrograde ejaculation. Transurethral surgery on the bladder neck may also lead to retrograde ejaculation, and patients undergoing deep pelvis surgery may experience disruption of the nerves responsible for erection and ejaculation.

Family History

It is important to identify whether other men in the family have had reproductive issues. A family history of infertility may increase the likelihood of finding an abnormality in the patient undergoing evaluation. Special interest should be paid to the maternal side as multiple genes, including the androgen receptor, are located on the X chromosome.

Social History

Many substances (even those consumed only socially) may negatively affect fertility. Tobacco use has been linked to reduced sperm production and function. Furthermore, semen parameters such as sperm density, motility, and morphology are all negatively impacted by consistent tobacco use.[29,30] However, research is inconclusive on the degree to which the use of tobacco translates into reduced fertility.[31]

Although alcohol, caffeine, and marijuana are known to negatively impact fertility in women, it is not known whether their use has a significant influence on semen parameters.[32] Research shows that use of

cocaine is detrimental to spermatogenesis, whereas chronic use of marijuana, alcohol, and narcotics has demonstrated a suppression of the HPG axis, which can impair male fertility.[33–36] Overall, it is best to counsel patients to limit alcohol consumption and avoid the use of illicit drugs and tobacco when trying for a pregnancy.

Exposures

It is well established that men who have undergone chemotherapy and/or radiation for cancer treatment are at risk for diminished sperm production or even permanent azoospermia. Although some men may only have temporary decline in semen parameters, many men will have permanent azoospermia.[37] Chemotherapy is a risk factor for infertility, but the risk is dependent on the dose and treatment regimen. Alkylating agents such as cyclophosphamide, chlorambucil, and ifosfamide are generally considered to be high risk for infertility. This risk is thought to be lowered when the cyclophosphamide equivalent dose (CED) is less than 4000 mg/m.[38] Platinum-based chemotherapeutic drugs are commonly used to treat testicular cancer and are known to significantly impair fertility.[39] Common platinum-based chemotherapeutic drugs are cisplatin and carboplatin. Decreased fertility in men is thought to occur with a total cisplatin dose greater than 400 mg/m^2 and a total carboplatin dose greater than 2 g/m^2.

Similar to chemotherapy, the risk of infertility after treatment with radiation depends on the duration, location, and dose of radiation. The testicle is generally not the object of treatment, but it may be impacted by scatter radiation from a nearby structural target. It should be noted that because it takes approximately 70 days to produce a mature sperm, the effects of radiation are not immediate and may not appear until 3 months posttreatment. Doses as low as 1–2 Gy have been reported to decrease the number of spermatozoa in the ejaculate, and doses of 4–6 Gy have resulted in severe ogliospermia.[40,41] Testicular shielding is recommended to reduce the risk associated with radiation treatment.

Certain prescriptions and over-the-counter medications may have an adverse effect on fertility and should be managed on an individual basis. The reactions can be mediated through central hormonal effects, direct gonadotoxic effects, influences on sperm production, and sexual function.[42] For example, 5 α-reductase inhibitors can adversely affect libido, decrease semen volume, and negatively affect sperm parameters, depending on dose and treatment duration.[42] Alpha-blockers can also result in reduced seminal emission and retrograde ejaculation, whereas antihypertensive and psychotropic agents have been shown to negatively affect sexual function and hormonal parameters.[42] The effects of antibiotics on sperm are largely unknown as existing data are limited and antiquated.[42]

Men with a history of prior use of exogenous testosterone or abuse of anabolic steroids are also at risk for suboptimal semen parameters. Exogenous testosterone suppresses the HPG axis via negative feedback. Many physicians are unaware that treating men with exogenous testosterone can compromise male infertility, whereas research demonstrates a link between exogenous testosterone and temporary azoospermia in most men.[43] One study showed that approximately 25% of urologists would treat men found to be hypogonadal and infertile with exogenous testosterone.[44] Fortunately, most of these men will be able to regain their sperm production within 1 year of cessation, but some may never completely recover.[45]

Occupational and chemical exposures can also be detrimental to sperm production. Although this hypothesis is difficult to study in humans, animal models have consistently demonstrated that environmental exposures pose a serious risk for fertility.[46–48] Pesticides may be a concern for agricultural workers, with a recent review and meta-analysis demonstrating a reduced fecundability ratio (FR) for both men and women.[49] Additionally, contact with heavy metals has been linked to abnormal semen parameters.[50]

Decreased sperm production is possible following exposure to extreme temperatures. For example, increased scrotal temperature and testicular hyperthermia adversely affect sperm production in men with varicocele and cryptorchidism. This knowledge has led to the development of behavioral modifications to avoid increased heat exposure and improve sperm production. Although there is no conclusive research showing that the use of a laptop or cell phone significantly affects sperm production, data

suggest that frequent use of hot tubs and saunas may be detrimental to sperm production and should be avoided when trying to conceive.[51]

Female Sexual and Reproductive History

An adequate assessment of a couple's fertility potential must include both partners. An evaluation for female reproductive risk factors will help to provide the clinician with a better overall picture of a couple's fertility. This is especially true for couples that pursue female infertility evaluation prior to male evaluation. It is important for the clinician to inquire about prior female fertility assessment. Common tests include hysterosalpingogram, pelvic ultrasound, and hormonal panels to check for potential imbalances.

A woman's age must be considered when evaluating fertility potential. Unlike males, a female's fertility is inversely related to her age; fertility potential for a woman in her late thirties is about half of the fertility potential of a woman in her twenties.[52,53] Advanced maternal age, defined as greater than 35 years of age, is associated with decreased fertility and increased risk of recurrent pregnancy loss, fetal anomalies, stillbirth, and obstetric complications.[54]

Female patients should be questioned regarding prior pregnancies, miscarriages, and elective terminations. Surgical interventions involving the reproductive organs may increase a woman's risk of infertility. These procedures include dilation and curettage, removal of uterine fibroid, or eradication of endometriosis. It is also important to assess for symptoms and other conditions that may indicate ovulatory abnormalities such as irregular menstrual period, polycystic ovarian syndrome (PCOS), endometriosis, and premature ovarian failure (POF).

Timing and Frequency of Intercourse

It is imperative that couples understand that conception generally coincides with ovulation of the egg into the fallopian tube. This "fertility window" is best defined as the 6-day interval ending on the day of ovulation.[55] Couples should be instructed to track the female partner's ovulation cycles to optimize their fertility potential. Methods for predicting ovulation include over-the-counter urine ovulation kits, monitoring basal body temperature, and examining cervical mucus changes. Sperm are generally viable within the female reproductive tract for 24–48 hours.[31] Based on this timing, intercourse is most likely to result in pregnancy when it occurs within the 3-day interval ending on the day of ovulation.[31] Other family planning studies have shown that the greatest likelihood of pregnancy occurred when intercourse took place 1–2 days prior to ovulation followed by a decline on the day of predicted ovulation.[55,56]

Recommendations regarding frequency of intercourse to achieve pregnancy are mixed. Sperm quality is known to be influenced by abstinence. Abstinence intervals longer than 5 days and shorter than 2 days have been shown to adversely affect sperm counts.[57] However, these data have not always translated into reduced pregnancy rates. Other family planning studies have demonstrated that couples engaging in daily intercourse had the highest fecundity rates followed by those having intercourse every other day.[55] The most important consideration for couples is sexual activity prior to and throughout the ovulatory period.

Coital Practices

There is no evidence that coital position during or after intercourse affects fecundability as studies have shown sperm in the cervical canal seconds after ejaculation.[31] However, in men with severe hypospadias, infertility may result from the inability to deposit semen into the most favorable location of the vagina/cervix. In these men, it is imperative to determine if they have normal erections and ejaculation.

Couples should generally avoid the use of lubricants due to their spermicidal effects. Lubricants known to be spermicidal include Astroglide®, K-Y Jelly®, Surgilube®, and saliva.[58–60] Even lubricants labeled as "sperm friendly" may disturb normal sperm function and should be avoided.[61,62] If a couple requires lubricant for intercourse, there are options that appear to be safe. Pre-seed® is a commercially available product that is designed for couples trying to conceive and appears to support sperm viability and function.[63]

Physical

A comprehensive evaluation for male infertility should include both general and genital examinations to assess for symptoms associated with underlying causes of infertility. The general examination includes observation of a man's general appearance and should assess for signs of obesity or cachexia. Research suggests that male obesity may have an adverse effect on fertility by altering hormone levels and sperm function.[64] The physician should also look for secondary sexual characteristics such as the presence of body hair, breast tissue, and muscle mass. These characteristics can serve as clues for the presence of infertility. For example, men with Klinefelter syndrome often have breast growth, decreased facial and body hair, reduced muscle tone, narrower shoulders, and wider hips.

The American Society for Reproductive Medicine Practice Committee states that the evaluation for male infertility should consist of a genital examination that includes examination of the penis, testes, scrotum, vas deferens, and epididymides.[3] Examination of the penis should note the location of the urethral meatus and any apparent penile irregularities or skin problems. The testes should be palpated for intratesticular masses, and the testicles should generally be similar in size, shape, and consistency. To ensure accurate measurement, an orchidometer or calipers can be used.

Following examination of the testes, the scrotum should be visually inspected and palpated superior to the testicle to assess for the presence of a varicocele. Varicoceles have been described as the most common correctable cause of male infertility as well as the most common cause of secondary infertility.[65] When assessing for varicocele, the patient should be examined in both the supine and standing positions. Varicoceles are typically more difficult to detect when supine and more obvious when the patient is upright and performing the Valsalva maneuver. A varicocele classically feels like a "bag of worms" within the spermatic cord, above the testicle. They are more commonly found on the left side which is believed to be due to the acute angle at which the left gonadal vein inserts into the left renal vein. Ancillary testing such as thermography, Doppler examination, radionuclide scanning, and spermatic venography should not be used to screen for varicoceles as the repair of subclinical varicoceles has shown inconsistent improvements in fertility.[3,66] Scrotal ultrasound may be useful in the setting of a difficult or equivocal physical examination.

The presence and consistency of the vas deferens and epididymides should be observed. The diagnosis of CBAVD is made on physical examination and scrotal exploration is not needed. Men with cystic fibrosis and men who are carriers of cystic fibrosis gene mutations classically have a complete absence of both vas deferens. However, it is important to remember that these men can have skip lesions as discussed in the section "Systemic Illness".

Conclusion

Male factors can contribute to couples' infertility in up to 50% of cases.[1,2] A proper evaluation of the male partner includes a thorough history and physical examination. This examination often identifies treatable or correctable problems that can help to optimize a couple's fertility potential.

There are many reversible male factors that can lead to natural fertility when treated. Other causes of infertility such as cancer and genetic abnormalities help to underscore the importance of a comprehensive male evaluation. Fortunately, most of these causes will be diagnosed during a thorough evaluation, and even when irreversible male factors are discovered, many couples are still able to overcome these obstacles due to technological advances in IVF and IVF/ICSI.

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The Role of Hormonal Profiles to Forecast Male Fertility Chances

Martin Kathrins and Craig Niederberger

Introduction

A thorough investigation into the causes of male infertility should include a hormonal evaluation. Although the criteria to diagnose hormonal dysfunction remain controversial, the integral role of androgens in spermatogenesis is well established. In fact, hormonal dysfunction among infertile men encompasses a broad spectrum of presentations—from asymptomatic genetic conditions to severe symptoms associated with hypoandrogenism. Thus, it is vitally important for physicians treating infertile men to understand the use of hormonal parameters to help guide the diagnosis and treatment of male infertility. We present a focused discussion of the role of hormonal parameters to predict fertility potential.

Hypothalamic–Pituitary–Gonadal Axis

The heterodimeric peptides follicle stimulating hormone (FSH) and luteinizing hormone (LH) are secreted by the anterior pituitary gland, or "master gland." The control of gonadotropin secretion is through the action of gonadotropin releasing hormone (GnRH), which is secreted in a pulsatile fashion from the hypothalamus through an interconnecting vascular plexus. Although further hypothalamic peptides have been discovered, which also control anterior pituitary gonadotropin secretion, including kisspeptin and gonadotropin inhibiting hormone, they have yet to secure a place in the clinical evaluation or treatment of male infertility.[1,2] The end-organ actions of LH and FSH are on testicular Leydig and Sertoli cells, respectively. FSH action on the germinal epithelium is requisite for spermatogenesis. LH is largely responsible for induction of testicular steroidogenesis via the Leydig cells, which is also necessary for spermatogenesis.

In addition to FSH and LH, the peptide hormones inhibin-B and activin also help control spermatogenesis. Inhibin-B is secreted by Sertoli cells and is antagonistically paired with FSH in a negative-feedback loop. Activin, although also produced by the germinal epithelium, is inhibited by inhibin-B action and exerts positive feedback on the anterior pituitary by increasing FSH secretion. A recent prospective trial of infertile men comparing FSH and inhibin-B found that the latter has slightly better potential to predict abnormal semen concentrations (FSH correlation coefficient [r] = −0.41, $p = 0.0007$ vs. inhibin-B; $r = 0.48$, $p < 0.0001$).[3] However, inhibin-B has not been widely adopted in routine clinical practice. Although inhibin-B may help determine spermatogenic status, activin has yet to achieve a clinically relevant diagnostic role.[4] Insulin-like factor 3 is an excellent biomarker for Leydig cell function; however, it also has yet to enter the routine clinical armamentarium.[5]

Interestingly, estradiol is the primary androgen that actually exerts negative feedback control on both the hypothalamus and anterior pituitary gland. Thus, through aromatization of testosterone both within the testicle and peripheral tissues, the male hypothalamic–pituitary–gonadal axis is a self-regulating system. Both the direct action of estradiol on the hypothalamus–anterior pituitary gland and aromatization of testosterone to estradiol are important pharmacologic targets in the treatment of fertility-related hormonal dysfunction.

Diagnosis and Physiology of Hypoandrogenism

The Endocrine Society defines hypoandrogenism as serum total testosterone below 280–300 ng/dL, based on repeated morning blood draws. Due to the increased avidity of testosterone for sex hormone binding globulin (SHBG) and albumin, only a small portion of testosterone is bioavailable. Thus, free testosterone (FreeT) is defined as the portion of total testosterone which is not loosely bound to albumin or tightly bound to SHBG. FreeT represents approximately 2% of total testosterone. Bioavailable testosterone (BioT) is the aggregate of FreeT in addition to that portion of testosterone that is only loosely bound to albumin. BioT represents up to 80% of total serum testosterone.[6] Much has been postulated about the relative accuracy of the various testosterone subcategories to reflect androgenization status, yet no confirmatory evidence exists to prefer one subcategory over another. Certainly, determination of either FreeT or BioT is warranted in the presence of medical conditions that predispose to abnormal serum SHBG or albumin concentrations, such as hepatic dysfunction. Regardless, The Endocrine Society recommends determination of FreeT or BioT in cases of equivocal findings with total testosterone.[7]

Published reference values for both FreeT and BioT are readily available. However, such reference values are based on direct laboratory assays that are fraught with inaccuracies and subject to high interlaboratory variability.[8] In our practice, we prefer to rely on validated calculations of BioT, based on morning assays of total testosterone, albumin, and SHBG, which are more reliable and reproducible. Vermeulen et al. presented such a validated formula to calculate serum testosterone fractions, further noting the lower-limit cut-off values for healthy young men. In our practice, we routinely use his cited reference value of 155 ng/dL for BioT as the final arbiter of adequate androgenization.[9]

Estradiol plays a vital role in spermatogenesis, underscored by the presence of estradiol receptors on all intratesticular cell types, including germ cells. Thus, the clinical use of the total testosterone-to-estradiol ratio (T:E) has become an important part of the hormonal evaluation of infertile men. A diminished T:E ratio has been associated with Leydig cell dysfunction and diminished bulk seminal parameters. Increased peripheral aromatization is also noted in men with elevated body mass index.[10] A T:E cut-off of less than 10—related to the normal lower-limit reference value for fertile men—may be used as a useful indicator of when hormonal therapy should be directed toward preventing peripheral aromatization rather than simply increasing intratesticular testosterone.[11] Thus, although selective estrogen receptor modulators may increase estradiol and cause more harm than good in such men, aromatase inhibitors may be used.

Perhaps the most important question from a fertility perspective is whether or not serum assays reflect the intratesticular milieu. Data from testicular aspirates of fertile men demonstrate that the intratesticular testosterone (ITT) concentration is approximately 10,000 times greater than the serum testosterone concentration.[12] The ITT concentration does appear to vary with LH pulsatility, with uncertain clinical implications.[13] The correlation between serum bioactive testosterone and intratesticular bioactive testosterone is quite low (r = 0.46, P = 0.03). However, the correlation between serum total testosterone concentration and ITT concentration is notably higher (r = 0.67, P = 0.03).[14,15] A diminished total testosterone-to-LH ratio may indicate Leydig cell dysfunction. Leydig cell dysfunction may, paradoxically, lead to pathologic Leydig cell clusters due to LH overstimulation.[16,17] Most importantly, the intratesticular concentration threshold across which spermatogenesis is impeded remains to be discovered. Underscoring this inconvenient fact is the 10%–15% of men who do not achieve significant oligozoospermia with experimental hormonal contraception. For responders and nonresponders, there is a significant overlap of ITT concentrations.[18]

However, although serum assays offer the possibility of an objective diagnosis, the correlation between such assays and the hypoandrogenic phenotype is tenuous. Correlation of serum total testosterone with validated questionnaires for symptomatic hypoandrogenism indicate that traditional cut-off values are poor predictors of the phenotype.[19] Furthermore, symptomatic "late-onset hypoandrogenism" has long been studied among older men, without a focus on the younger, healthy hypoandrogenic patients who may have fertility-related hormonal dysfunction.[20]

The relationship between ITT and spermatogenic status appears to be quite complicated. Retrospective series of infertile men with normal-range FSH found that ITT concentrations may actually be elevated

relative to fertile controls.[21,22] Such a finding raises the possibility of downstream androgen receptor dysfunction. Unfortunately, polymorphism analyses of the human androgen receptor gene have not been successfully correlated with any measure of fertility potential.[23] Alternatively, retrospective studies of men undergoing a second microsurgical testicular sperm extraction—after one prior failed surgery and who were subsequently treated with exogenous gonadotropins—found that those men with lower pre-treatment ITT were more likely to respond to the hormonal therapy and go on to a successful second sperm extraction. This would seem to suggest that lower ITT in the setting of azoospermia due to spermatogenic dysfunction (ASD) may be a pathologic state, responsive to therapy.[24] Although chromosomal and genetic mutations explain a portion of men with ASD, there appears to be a subset of patients who owe their presentation to severe intratesticular hypoandrogenism. Interestingly, data from animal studies indicate that the androgen receptor—localized almost exclusively to the Sertoli cell—is necessary for completion of spermatogenesis, the absence of which leads invariably to spermatogenic maturation arrest pathology.[25,26] Thus, hormonal dysfunction clearly leads to impaired fertility, but our ability to accurately diagnose such dysfunction using existing clinical tools is limited.

Association between Hormonal Dysfunction and Fertility Potential

The American Society of Reproductive Medicine and the American Urological Association express similar recommendations in regard to the hormonal evaluation of infertile men.[27,28] The authors of those recommendations suggest that a basic hormonal evaluation—consisting of total testosterone and FSH—only be performed in the setting of oligozoospermia (less than 10×10^6 sperm per millileter), sexual dysfunction, or "other clinical findings suggestive of a specific endocrinopathy." Sigman and Jarow published a large retrospective multicenter series of infertile men and found that only 9.6% of men presented with an endocrinopathy after repeated testing. However, they noted that only 1.7% of all men presented with a "significant" endocrinopathy, after excluding men with hypergonadotropic hypoandrogenism. If employing a screening cut-off of sperm concentration less than 10×10^6 sperm/mL, only a solitary patient in their entire series actually had a "significant" endocrinopathy. Unfortunately, the article does not detail their definition of hypoandrogenism nor much of their criteria for a "significant" endocrinopathy.[29]

However, such a low prevalence of hypoandrogenism among infertile men has not been replicated in other studies. Indeed, Sussman et al., in a single-institution review of 120 infertile men, found that 45% of men with ASD, 43% of men with oligozoospermia, and—interestingly—35.3% of men with normozoospermia demonstrated hypoandrogenism based on the Endocrine Society diagnostic guidelines. Their internal control—men with obstructive azoospermia after vasectomy—revealed that only 16.7% of these men were diagnosed with hypoandrogenism, aligned with the prevalence of hypoandrogenism in the general population.[30] A similar prevalence of hypoandrogenism among men with ASD was noted in another retrospective series.[31] Patel et al., using cut-off values of 155 ng/dL bioavailable testosterone or, in the absence of BioT, 300 ng/dL total testosterone, found that 34% of men with ASD presented with hypoandrogenism. Importantly, they again demonstrated that up to 44% of men with normozoospermia may harbor hypoandrogenism. They ultimately found that serum testosterone was a poor predictor of sperm concentration.[32]

Differentiating between hypogonadotropic and hypergonadotropic hypoandrogenism is important when evaluating infertile men. Congenital hypogonadotropic hypoandrogenism presents with absent or delayed puberty. Although such patients are often diagnosed early in life, a comprehensive developmental history in the evaluation of adult men is of paramount importance. For patients with a congenital presentation, it is also recommended to screen for underlying causative medical conditions, including genetic conditions (e.g., Kallman syndrome associated with anosmia), pan-hypopituitarism, and hyper-prolactinemia. Adult-onset idiopathic hypogonadotropic hypoandrogenism presents after normal pubertal development but with similarly depressed gonadotropin levels. Such men, properly diagnosed, often present with ASD and are invariably responsive to gonadotropin replacement therapy.[33] Opiate-induced hypogonadotropic hypoandrogenism is an underappreciated cause of infertility; if possible, weaning of the offending medications should be encouraged.[34]

Exogenous testosterone replacement therapy or anabolic-androgenic steroid abuse has become an increasingly problematic cause of hypogonadotropic hypogonadism and infertility. Surprisingly, a recent survey of 387 American urologists found that 25% of respondents used testosterone replacement therapy as empiric treatment for male infertility.[35] Indeed, "designer" anabolic steroids—available over the counter—are a very common cause of hypoandrogenism among men seeking care for infertility.[36] In addition to significantly diminished gonadotropin levels due to suppression of the hypothalamic–pituitary–gonadal axis, such patients may present with testicular atrophy and gynecomastia. Although removing the offending agent is the treatment of choice, reports of return of normal spermatogenesis after 3–5 years of continued use are few and far between. Such men may require more aggressive hormonal therapy.[37]

Hypergonadotropic hypoandrogenism, or testicular failure, has a number of underlying causes. The most common cause of fertility-related hypergonadotropic hypoandrogenism is Klinefelter syndrome, caused by a supranumery X chromosome. Such patients are also notable for a diminished T:E, due to hypothesized increased intratesticular aromatase activity.[38] Prior chemotherapy—particularly platinum-based therapy—may induce hypoandrogenism via Leydig cell toxicity; however, only higher treatment dosages appear to cause long-term irreversible damage.[39] Serum assays of LH are also useful predictors of treatment response to selective estrogen receptor modulator medications, such as clomiphene citrate. One single-institution retrospective series found that men with pretreatment LH levels greater than 6 IU/L were less likely to achieve adequate androgenization after clomiphene citrate therapy.[40]

Gonadotropin assays are also valuable in determining spermatogenic status. Although the published reference ranges for FSH are quite variable, Gordetsky et al. found—based on a large retrospective series—that an FSH cut-off value of 4.5 IU/L signifies an increased risk of abnormal semen concentration (odds ratio 3.66, 95% confidence interval [CI] 2.08—6.44). Perhaps the most reliable use of gonadotropin assays is in the differentiation of azoospermia due to either obstruction versus spermatogenic dysfunction, respectively. Schoor et al. found that—using an FSH cut-off value of greater than 7.6 mIU/L—76% of patients with ASD were correctly diagnosed without the need for a diagnostic testicular biopsy. When combined with a testicular longitudinal axis determination, the ability to accurately diagnose ASD is 89% and obstructive azoospermia is 96%.[41] The use of FSH as the best biomarker to categorize azoospermic men was further validated in other retrospective series—with variable FSH cut-off values used. [42–44]

Use of Hormonal Profiles to Predict Surgical and Medical Treatment Outcomes

Traditionally, elevated FSH has been considered a poor prognostic indictor for successful surgical sperm extraction. Perhaps this was due to the use of now defunct blind testicular sperm extraction techniques to obtain sperm from men with ASD, which did not account for the heterogeneous foci of spermatogenesis in the testes of such men. However, Tournaye et al., in 1997, published a series of random incisional testicular extraction procedures—stopping only once sperm was obtained—and demonstrated that FSH was not highly predictive of successful sperm retrieval.[45] Subsequently, Ramasamy et al. published a large single-institution series from Cornell of microsurgical testicular sperm extraction procedures and also found that FSH was a poor predictor of successful surgical sperm retrieval. Of 792 men in their series, three successful sperm retrievals were performed in men with FSH levels greater than 90 IU/mL. Interestingly, they found the subset of men with an FSH less than 15 IU/mL to have comparatively lower successful sperm retrieval rates. The authors hypothesized that the subset of men with diffuse maturation arrest may present with normal gonadotropins and normal size testes, making this small subpopulation of men with ASD quite difficult to accurately stratify preoperatively.[46] An expanded cohort from Cornell was subsequently used to formulate a neural computational model to predict surgical sperm retrieval rates from microsurgical testicular sperm extraction. Again, FSH was not a significant predictor of surgical outcome.[47]

Gonadotropin assays may also be a useful guide to predict surgical outcomes among men with clinically significant varicoceles. Traditionally, patient counseling regarding fertility outcomes after

varicocelectomy has focused only on varicocele grade and baseline seminal parameters. Kondo et al. performed a retrospective analysis of 97 men with oligozoospermia and a mixture of left and bilateral clinical varicoceles. On multivariate analysis, the authors found that a lower FSH was significantly associated with a positive response to varicocelectomy (odds ratio 0.881, 95% CI 0.779—0.997, $P = 0.04$).[48] Similarly, Yoshida et al., in a retrospective analysis of 168 infertile men with left-sided only varicoceles, demonstrated that an FSH cut-off of less than 11.7 mIU/mL was associated with improved postsurgical outcomes.[49] Although still controversial, varicocelectomy in the setting of ASD is more likely to subsequently produce sperm in the ejaculate—thereby avoiding testicular sperm extraction—if the preoperative FSH is less than 10.1 mIU/mL.[50] Thus, significantly elevated FSH values reflect severely impaired germinal epithelium, unlikely to recover after varicocelectomy surgery.

Conclusion

It is clear that a high proportion of men presenting with infertility may have some degree of hormonal dysfunction. However, the relationship between basic serum androgen assays and semen parameters is quite complicated. That is underscored by the difficulties inherent in correlating the intratesticular milieu with serum assays and testicular spermatogenic pathologies. Yet, by interpreting serum testosterone assays with gonadotropins, much important clinical data can be gleaned with direct impacts on fertility outcomes in regard to diagnostic categorization and surgical outcomes.

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The Application of Genetic Tests in an Assisted Reproduction Unit: Karyotype

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Introduction

Infertility is a condition of disease character with multifactorial etiology. According to the World Health Organization (WHO),[1] infertility is defined as the inability of a sexually active couple at reproductive age to achieve pregnancy within 1 year of unprotected intercourse. Data on the prevalence of infertility vary considerably between 8%[2] and 25%,[3] of which about 15% of the individuals are seeking medical assistance and 5% ultimately remain childless.[3] The prevalence of infertility is 30%–50%, more or less equally distributed between women and men.[4] An estimate of 7% of all men are confronted with fertility problems during their reproductive lifetime, which renders male infertility a problem with even higher prevalence than a common disease, diabetes mellitus, with an overall estimate of 2.8% in the year 2000 and 4.4% in 2030.[5,6]

The main causes for male infertility include anatomical and developmental defects, ejaculatory failure, environmental toxicity, dysfunctional spermatogenesis, endocrine and immunological disturbances, systemic and lifestyle diseases, or abnormal sperm functions. Thus far, the diagnostic methods of choice for male infertility were the clinical examination of the patient as well as a standard semen analysis, which still forms a cornerstone of andrological diagnosis but is incomplete and neither properly predicts the fertilization outcome,[7,8] nor provides sufficient information about the functional capacity of the spermatozoa, leaving it difficult to discriminate between fertile and infertile subjects as approximately 40% of infertile men present with normal semen parameters.[9,10] Therefore, in the last two decades, standard semen analysis was complemented by functional sperm parameters such as acrosome reaction, zona pellucida binding, mitochondrial membrane potential, or sperm nuclear DNA fragmentation, including *omics* analyses as molecular techniques.[11,12] Yet, although the latter approaches offer novel diagnostic avenues and narrow the gap, both proteomic and genomic methodologies are still lacking the indubitable identification of markers that meet all the criteria for a good clinical marker as well as the necessary validation. Therefore, the implementation of these novel techniques into clinical routine will still take some time.[12] Consequently, the pregnancy success rates for in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) remain at 29%–33% relatively low[13] and have not improved significantly during the past 30 years.[14] This is partly due to the fact that standard semen analysis and sperm functional tests do not detect the cause of male infertility including the related sperm abnormalities in about 20%–40% of the patients, leading to high rates of idiopathic infertility.[15,16] This high prevalence of idiopathic male infertility ultimately leads to high cost, and frustrating and traumatic experiences on the part of the patients as these patients have to go through many unsuccessful treatment attempts.

Idiopathic oligozoospermia and azoospermia are often associated with genetic and epigenetic abnormalities, including numerical and structural chromosome abnormalities.[17] Particularly in these patients, the most invasive assisted reproductive technology (ART), ICSI, which bypasses all physiological barriers, is used for these patients to father a child. However, the use of genetically compromised sperm for assisted reproduction has shown numerous adverse consequences, including abnormal embryo development, higher miscarriage rates, and birth defects of the progeny.[18] Considering that the process of spermatogenesis involves the concerted action of an estimate of 2300 genes to enable fertility in

healthy men,[19] of which only about 30 genes are located on the Y chromosome,[20,21] this not only requires a better understanding of the complex processes involved, but also an extended clinical examination, including genetic testing in men with unexplained infertility to determine the cause of the infertility and the clinician being able to provide appropriate counseling and management of the disease.

Infertile men usually present with impaired spermatogenesis, structural genital abnormalities, smaller testes, hypogonadism, and/or abnormal sperm functions.[20] Clinically, an estimate of 10%–15% of infertile men present with genetic abnormalities, which can be grouped in four categories, namely chromosomal defects in somatic cells, gene mutations in somatic cells, sperm chromosomal abnormalities, and epigenetic disorders.[22] In this context, azoospermia, obstructive or nonobstructive, represents a major group of patients with unexplained infertility amounting to 10%–20% of these cases,[23,24] of which 21%–29% can genetically be explained.[25] Considering this high prevalence and the elevated risk of serious adverse consequences, genetic testing in these patients is strongly recommended to identify genetic conditions that might be passed on to the next generation, as well as impacting the ability of sperm retrieval using microsurgical extraction techniques.[26,27]

For infertile men, genetic tests are recommended in cases of azoospermia during the diagnostic workup, severe oligozoospermia ($<10 \times 10^6$/mL) during the diagnostic workup and prior to any form of assisted reproduction, and in cases of moderate oligozoospermia and normozoospermia if no pregnancy was achieved after 1 year of regular unprotected intercourse.[26] Other authors even recommend mandatory cytogenetic analyses in cases of severe oligozoospermia and nonobstructive azoospermia.[28,29] However, in light of the 10% of female infertility to be attributed to genetic factors,[26] genetic testing should also be conducted in women presenting with amenorrhea and oligomenorrhea with hypergonadotropism during the diagnostic workup and prior to assisted reproduction, in cases of hypogonadotropic hypogonadism during the diagnostic workup, apparently normal cases after 1 year of regular unprotected intercourse or prior to assisted reproduction, and in cases with recurrent pregnancy loss during the diagnostic workup.

Among the genetic abnormalities, chromosomal defects account for up to about 15% of the cases,[29] of which Klinefelter syndrome, chromosomal translocations (autosomal, sex chromosome, and Robertsonian's translocations), inversions, and deletions are common and the Klinefelter syndrome with 0.2% of all male newborns and 11% among those of azoospermic fathers being the most prevalent aneuploidy form.[30,31] Klinefelter syndrome (47,XXY) is the most common cause of azoospermia. The phenotypic appearance of Klinefelter patients varies greatly, ranging from normal virility to that of severe androgen deficiency with female hair distribution. This is due to the high (10%–20%) percentage of mosaicism (47,XXY/46,XY).[32] Even karyotypes such as 48,XXYY, 48,XXXY, and 49,XXXXY have been found,[32–34] with the latter two being debatable whether they should be regarded as Klinefelter syndrome variants since they differ significantly from the appearance of normal Klinefelter patients.[35] Yet, the vast majority of Klinefelter patients have testicular sizes less than 10 mL and significantly elevated follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels. Although up to 8.4% of nonmosaic Klinefelter patients present with spermatozoa in their ejaculates due to some focal spermatogenesis, azoospermia is the norm,[32,36] with about 93% of the patients azoospermic. The treatment option of choice in these patients is testicular sperm extraction (TESE) as testicular sperm can be recovered in about 50% of the patients,[37] offering them the opportunity of having their own genetic children. Although the sperm aneuploidy rate in Klinefelter patients appears to be elevated,[38] there is reason to believe that the risk for the progeny is small as less than 1% of XXY-pregnancies after TESE–ICSI with sperm from nonmosaic Klinefelter patients have been reported.[35] Nevertheless, future parents need to be counseled and genetic problems in the offspring due to assisted reproduction must be avoided.

A number of different genetic tests are available including cytogenetic analysis, Yq microdeletion analysis, or various mutation analyses, e.g., for cystic fibrosis transmembrane conductance regulator (CFTR) or Kallmann syndrome. Cytogenetic analyses describe basically two different types of tests, namely fluorescent in situ hybridization (FISH) analysis and karyotyping, for which three approaches are to be mentioned: the classic karyotype method involving the collection of heparinized blood samples with the isolation of lymphocytes, the karyotyping of spermatozoa using the hamster ovum penetration test, and the recently developed method of molecular karyotyping of single sperm cells by array-comparative genomic hybridization.[39] The FISH analysis combines the classic karyotype methods with in situ hybridization using a fluorescent DNA probe.

This chapter aims to summarize the genetic testing methodologies used for genetic screening of infertile males, focusing on karyotype analysis, sperm FISH, and polymerase chain reaction–based testing, as well as multiplex ligation-dependent probe amplification (MLPA) for Y chromosomal evaluation. This chapter also summarizes the cutting-edge genetic testing methodologies using oligonucleotide-array–based comparative genomic hybridization (array-CGH) and whole-genome sequencing, which allow analysis at a nucleotide-level resolution.[40–42]

Cytogenetic Analysis

Cytogenetic abnormalities are an important cause of male infertility, accounting for 10%–15% of infertility cases.[43] These genetic causes of spermatogenetic impairment include both chromosomal abnormalities and single-gene mutations,[40] which influence many physiological processes involved in male reproduction, including hormone homeostasis, spermatogenesis, and quality of sperm.[44] For this reason, the identification of genetic factors of male infertility is important for the appropriate assistance of infertile couples.[43,45] Consequently, genetic screens are routinely included in the diagnostic work-up of infertile males; different guidelines have been proposed for the appropriate use of these genetic tests in cases of low sperm count and/or motility, or before commencing an assisted reproduction program.[26,45,46]

Karyotype Analysis

Even though the first genetic-type tests evaluating an individual's DNA makeup were reported during the 1950s,[47,48] these initial karyotype analyses did not assess specific regions of DNA, and they were not used in clinical settings.[40] On the other hand, karyotype analysis in male fertility was already in use by the 1960s.[49] Karyotype analysis is a cytogenetic study evaluating the number and appearance of chromosomes using light microscopy for structural defects. Although karyotype analysis remains in routine use for the detection of structural chromosomal abnormalities in infertile men, a major shortcoming is the inability to detect DNA changes smaller than 4 Mb, and it is also labor intensive and time consuming to perform.[40]

The usefulness of karyotype analysis was later improved by the staining of each chromosome in metaphase with Giemsa stain (also known as G-banding), generating a unique signature pattern for each chromosome. This allows for the identification of chromosomal translocations, deletions, and insertions for each chromosome to be identified.[50] Despite the fact that karyotype analysis represents a very superficial look at genetic material, it can still provide valuable information to infertile couples,[40] and a significant percentage of both male and female infertility has been linked to genetic abnormalities identified by karyotype analysis.[51–55]

The sperm penetration assay (SPA) allowed for the first analysis of chromosomes from human sperm.[56–58] In this in vitro assay, the zona pellucida is removed from hamster oocytes and the oocytes are allowed to fuse with human sperm.[56,59] In essence, the SPA is used to evaluate male fertility potential by determining sperm's functional capacity. This is done by measuring the spermatozoa's ability to undergo capacitation, acrosome reaction, fusion and penetration through the oolemma, and decondensation of the male nucleus within the cytoplasm of the hamster ova.[60] Importantly, the SPA has been thought to be superior to seminal fluid analysis in predicting both fertility and infertility,[61,62] and many variations of the test have successfully been used in the clinical setting.[62] Disconcertingly, false-negative results (men whose spermatozoa fail the SPA but successfully fertilize human oocytes in vitro or in vivo) have been reported,[63–65] and the validity and reproducibility of the SPA has not been established yet.[60] Due to the latter problems, and the fact that the SPA is time consuming, relatively expensive, and highly dependent on skilled expertise, researchers have proposed that the assay should not be used to evaluate fertility potential.[60]

Comparative genomic hybridization (CGH) enables the screening of an entire genome for genetic modifications. CGH uses differentially fluorescent-labeled test and normal DNAs that are hybridized to metaphase chromosome spreads. The color and intensity of fluorescence are then interpreted to identify

regions of copy number gain or loss.[66] The clinical usefulness of CGH is limited by its labor intensiveness and limited resolution of 5–10 Mb, which is similar that of a standard karyotype.[67,68]

A modified version of CGH, using a microarray platform (array-CGH), has significantly increased resolution, with current assays resolving to less than 1 kb,[69] while screening the entire chromosome complement for microscopic and submicroscopic imbalances.[39,70] When screening for these chromosomal abnormalities, array-CGH, which also results in more precise mapping of aberrations, is now used as a first step and no longer as an adjunct test to standard karyotype analysis.[70] Although array-CGH is currently used in both research and diagnostic clinical settings, including prenatal diagnosis and preimplantation genetic diagnosis, the procedure has only recently been applied to single sperm to obtain a "molecular karyotype" allowing for the analysis of the complete genome of a single sperm. Here, array-CGH has been used to identify aneuploidies and chromosomal alterations with high resolution and accuracy.[39] Importantly, even though array-CGH cannot detect balanced rearrangements such as translocations, insertion, and inversions, the procedure still provides distinct advantages over conventional and molecular cytogenetic analysis.[70]

Fluorescent In Situ Hybridization Analysis

FISH combines classic karyotyping with in situ hybridization using fluorescent DNA-specific probes for the identification of specific DNA sequences on chromosomes in intact cells[71–74]; sperm FISH uses two or more DNA probes on decondensed sperm nuclei.[39,75,76] In a clinical setting, sperm FISH is used in cases of recurrent pregnancy loss, because even normozoospermic male partners in couples with recurrent pregnancy loss have high rates of sperm aneuploidy,[77–79] as well as in men with severe teratozoospermia or oligoasthenoteratozoospermia, because it can define meiotic defects. This helps physicians and counselors direct counseling efforts.[40,80] FISH analysis can also be used to assess sperm hampered by abnormalities in motility or other aspects of fertilization (see review, Ramasamy et al.[81]).

Currently, sperm FISH is limited by the probes available, which focus on chromosomes X, Y, 13, 18, and 21 because aneuploidies of these chromosomes are associated with viable offspring; targeting other chromosomes is feasible, but prohibited by high experimental costs.[81] Another problem with FISH is poor resolution, typically limited to several megabases. "Fiber FISH," a variant of FISH, uses metaphase chromosomes that have been mechanically stretched, improving resolution to several kilobases.[82,83] To date, this variation on FISH has not been used in sperm studies.

Yq Microdeletion Analysis

Restriction fragment length polymorphism (RFLP) analysis was first used to confirm a Y chromosomal loss that had initially been identified on karyotype analysis in male fertility evaluation.[84] In combination with other haplotyping methods, RFLP analysis was then used to identify three Y chromosomal haplotypes. Interestingly, sperm concentrations varied between these haplotypes and certain haplotypes were more commonly linked with infertile men.[85] However, in the end, because of a lower frequency of polymorphisms than that found on other chromosomes, RFLP analysis proved inadequate for the evaluation of the Y chromosome.[40,86]

Polymerase chain reaction (PCR) is a rapid method that replaced RFLP analysis for detection of submicroscopic Y chromosome deletions, which are also undetectable by conventional cytogenetic analysis[29,87]; multiplex PCR is normally used to amplify the AZFa, AZFb, and AZFc loci in the q-arm of the Y chromosome.[29,88] Since deletions of the long arm of the Y chromosome (Yq) are found in about 10%–20% of men with azoospermia or severe oligozoospermia,[40,89–93] PCR is crucial in identifying male infertility. Single sperm typing using PCR is a complex, time-consuming technique, but it is still useful for specific studies on recombination in delimited areas of the genome.[94,95]

Multiplex PCR recommended by the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) best practice guidelines for detection of Y chromosome aberrations[96] is not sensitive enough to detect the most commonly reported partial deletions or duplications of the Y chromosome[97]; the MLPA has been proposed for this purpose. The MLPA is a sensitive technique routinely used for copy number analysis in various syndromes and diseases. The assay is used

for relative quantification of up to 50 different nucleic acid sequences in a single reaction tube, which obviously minimizes experimental time and cost.[98] When screening for potential Y chromosome deletions, the MLPA has numerous advantages over PCR-based methods as it allows the sensitive detection of almost all microdeletions or microduplications of various sizes.[41,97,99,100]

Sequencing

High-level resolution of molecular karyotype, mutations, deletions, and amplifications of DNA is limited with the currently used molecular techniques, including CGH and array-CGH. Resolution at the nucleotide level is, however, possible, using whole-exome (WES) and whole-genome sequencing (WGS) approaches.[40] Whereas WGS refers to the sequencing of all bases within the genome, WES involves sequencing of the expressed regions of the genome, or exons, only.[101] Obviously, WES remains more cost effective than WGS and could potentially result in a higher likelihood of identifying significant mutations considering that approximately 85% of disease-causing mutations are believed to reside in gene and functional coding regions.[102,103] Thus, WES could potentially identify the cause of many rare genetic disorders, as well as predisposing variants in more common diseases,[101] including male infertility. As an example, the CFTR, an important gene studied in the field of male infertility, is sequenced in men with obstructive azoospermia due to congenital bilateral absence of the vas deferens, a genital manifestation of cystic fibrosis.[101,104] Next generation sequencing (NGS), also known as high-throughput or second-generation sequencing, is the umbrella term used to describe a number of different modern sequencing technologies that can be done within a single day.[105–107] These exciting new technologies translate into savings in experimental cost and also have applications that are immediately relevant to the medical field.[106] Although NGS has not yet been used in the study of male fertility, and very few of the more than 200 genes associated with male infertility in mouse models and humans are clinically assessed in patients today,[108] it is still clear that sequencing potentially has a major role to play in the field of male infertility.

Conclusions

The increasing prevalence in male infertility and the continuous development of assisted reproduction techniques necessitates the need to better understand the causes of male infertility. Understanding the genetics of sperm damage and the continuous development of the genetic and molecular techniques used for sperm evaluation and selection are important advances that will lead to the optimization of the diagnostic and therapeutic management of male and couple infertility, especially for idiopathic infertile couples and those undergoing ART cycles. Generally, DNA damage in sperm cannot be detected by routine cytogenetic and molecular protocols, which lead to the development of various techniques such as Comet assay, sperm chromatin dispersion assay (SCD), in situ terminal deoxynucleotityl transferase mediated dTUP nick end labeling (TUNEL), or sperm chromatin structure assay (SCSA).[29,45] This review summarizes more recent techniques/assays developed to circumvent many of the shortcomings of these earlier cytogenetic and molecular protocols. Faced with such a wide selection of all of these newly developed techniques and diagnostic assays, it is critical to know what tests are already routinely used in the clinical setting and those that are likely to be used in the near future. Clearly, modern genetic/molecular testing technologies will make rapid whole-genome assessment of the infertile male possible. This will result in a future where the evaluation of male infertility could result in a personalized diagnosis and treatment for each infertile male.

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5

Genetic Testing of Y-Chromosome Microdeletion

Jason C. Chandrapal and James M. Hotaling

Fifteen percent of reproductive-aged couples worldwide are diagnosed with infertility after a 1-year period of failed conception.[1] With male factor infertility occurring in approximately one-half of these cases, the male factor plays a prominent role.[2] Because male infertility can have multiple causes, the American Urological Association (AUA) recommends a detailed medical and reproductive examination with semen analysis before moving forward with more specific testing.[3] If no clear explanation of infertility from the history or physical examination is available and the semen analysis reveals a sperm count of less than 5 million/mL, genetic testing is recommended.

Genetic disorders account for up to 30% of infertility cases and consist of two major categories: single gene point mutations or single nucleotide polymorphisms (SNPs) and chromosomal abnormalities.[4] Single gene mutations are seen in cystic fibrosis when deletion of a single phenylalanine at position 508 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene can cause congenital bilateral absences of the vas deferens (CBAVD).[5] Chromosomal abnormalities resulting in infertility occur in about 14% of men with azoospermia and oligozoospermia and consist of structural (Kallman syndrome, mild androgen insensitivity, and Y-chromosome microdeletion) or numerical (Klinefelter syndrome) abnormalities.[6] Although these disorders represent the most common genetic disorders affecting fertility, there are still many unexplained cases.

Y-chromosome microdeletions (YCMD) are the second most frequent genetic cause of infertility in men behind Klinefelter's syndrome. The association between YCMD and infertility is based on the difference in prevalence of YCMDs between fertile and infertile males. Within the general population YCMD occurs in 1 in 4000 men, whereas men with azoospermia have a mean prevalence of 9%, depending on the population (Table 5.1).[7,8] Men with severe oligozoospermia may also carry these deletions, in which YCMDs affect 2% of this population (Table 5.1).[8] Deletions generally occur within one of three sequences of the long-arm section (Yq) of the male-specific region Y chromosome (MSY) and have a variable effect on sperm production, depending on the size and location of the deletion.[9–12] Because YCMD testing can help determine the probability of finding sperm on testicular extraction, guidelines recommend YCMD testing as standard workup for men with nonobstructive azoospermia or severe oligozoospermia.[3,7,13]

History of the Y Chromosome

The first step in differentiation of the X and Y chromosome occurred with the acquisition of a testis-determining gene. This was followed by a series of large inversions on the Y chromosome that disrupted the homology between the two chromosomes and prevented recombination with X chromosome; the nonrecombination region of the Y chromosomes was later renamed the MSY. In the absence of crossing over, the Y chromosome was subjected to deletions and gene loss, thus shrinking the Y chromosome to its present day form. The paucity of genetic material compared with the X and autosomal chromosomes led many early biologists to believe that the Y chromosome was useless and on the way to extinction.[14] This reduction in size was originally thought to be a random process; however, we now believe that these deletions were purposeful. Evolutionary pressures removed nonvital genetic material producing a truncated chromosome with a high concentration of active genes. The persistence of this genetic information means that the genes from the Y chromosome are necessary to the organism and the reduction of junk DNA makes the Y chromosome one of the most efficient chromosomes in the genome.

The first research breakthrough for the Y chromosome was the discovery of the testis-determining gene. Early genetic experiments, conducted in XX males and XY females, defined a 300-kb segment within the short arm of the Y chromosome (Yp); this would later be known as the sex-determining region of the Y chromosome (SRY) gene and be responsible for male gender differentiation.[15,16] The next significant milestone in Y chromosome research was the link to spermatogenesis. The first study to describe this relationship was Tiepolo et al. in 1976, which examined the karyotype of 1170 men; within this cohort they found six men with azoospermia and normal phenotypes. This group of azoospermic men had a consistent deletion that extended from the distal euchromatic band (Yq11) to the entire heterochromatic region (Yq12).[17]

Mapping the Y chromosome significantly improved the quality and specificity of deletion detection. Early genetic studies of YCMD, using light microscopy, would only reveal the presence of broad deletions, without specifics of deletion boundary or content. Additionally, these studies were restricted to large deletions because smaller deletions were not detectable by light microscopy. The creation of a physical or deletion map of the Y chromosome would help define specific deletion sequences and target genes vital to spermatogenesis. In 1986, Vernaud et al. used DNA hybridization techniques in individuals with Y chromosomal abnormalities to create the first Y chromosome deletion map.[18] This initial map divided the Y chromosome into seven distinct regions with intervals 1–3 on the short arm (Yp), 4 on the centromere, and 5–7 on the long arm (Yq) (Figure 5.1).

TABLE 5.1

Large Studies Comparing the Prevalence of YCMD in Patients with Infertility

Study	Year	Location	Cohort Size	YCMD %	Azoospermia + YCMD %	Severe Oligozoospermia + YCMD %
Ferlin et al.[85]	2007	Italy	3073	5	8	3
Kumtepe et al.[86]	2009	Turkey	1935	8	10	2
Stahl et al.[52]	2011	USA	1591	9	10	2
Zhang et al.[68]	2013	China	1738	9	6	2
			Average	**8%**	**9%**	**2%**

Note: Severe oligozoospermia was defined as <5 million sperm/mL.

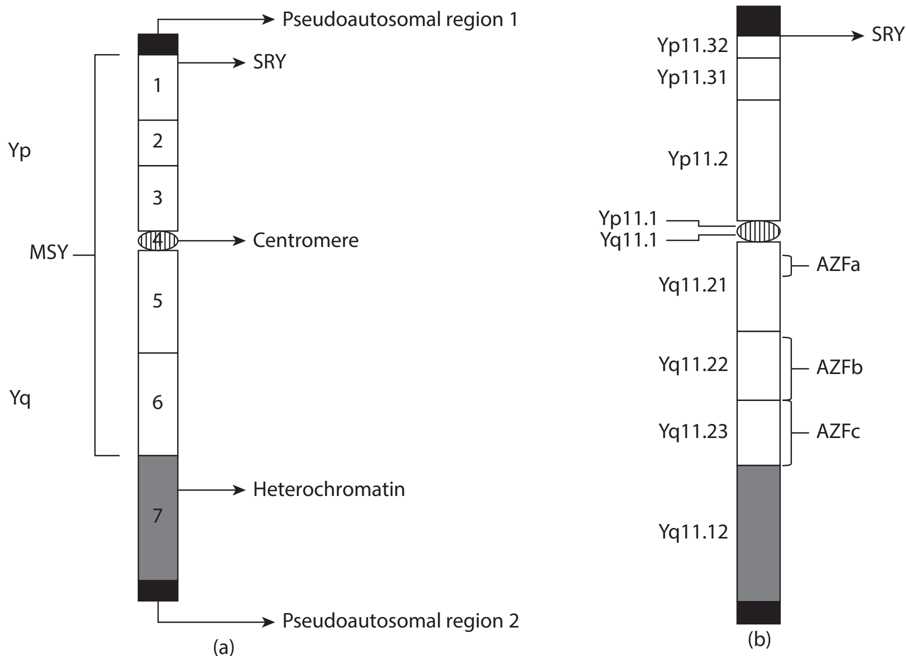

FIGURE 5.1 Y-chromosome maps. (a) Vernaud map featuring seven distinct intervals. (b) Cytological banding Y-chromosome map.

The use of sequence tagged sites (STS) to detect specific deletions expanded the Y-chromosome map. STS are sequences of genomic DNA amplified by polymerase chain reactions (PCR). These sequences may be gene specific or overlap anonymous regions of the Y chromosome.[19] Utilization of STS deletion detection allowed for the expansion of the number of intervals within the Y chromosome, creating a more detailed map. In 1992, Vollrath et al. used STS to characterize the deletion map of the euchromatic portion, in which intervals 1–6 from the Vernaud map were expanded to 43 intervals.[20] That same year, using a library of yeast artificial chromosome (YAC) clones to clone Y-chromosome DNA, Foote et al. used 160 STS to create a Y-chromosome map defining 127 intervals.[21] Once a detailed map became available, molecular studies could definitively demonstrate the central role of the Y chromosome in spermatogenesis.[9–11,22]

Current research has shifted focus from the reproductive implications of the Y chromosome to the nonreproductive effect of these genes. A majority of genes on the Y chromosome are not restricted to the chromosome and found on either the X chromosome or autosomes, with variable expression. The exact role of these nonspecific genes is unclear. Originally, these gene products were considered irrelevant; however, current theory is that they play a much larger role. Evidence of this is seen in the standard phenotypic traits of individuals with sex chromosome aneuploidies such as Klinefelter syndrome, XXY males, and individuals with sex reversal (XY females and XX males).[23–28] Apart from their influence on phenotypic appearance, genes on the Y chromosome may impact the overall health. Male factor infertility has been associated with a higher risk for clinically significant prostate and testicular cancer.[29,30] Hanson et al. expanded on this association between male factor infertility and cancer and reported that men with oligozoospermia or low sperm motility had an increased risk for all site cancer.[31] Infertility has been found not only to impact the health status of the individual, but it may also affect their relatives. Pedigree analysis of men with azoospermia found that the fathers/grandfathers of these individuals had a higher risk of early cardiovascular-related, cancer-related, and overall mortality compared with fathers/grandfathers of normozoospermic men.[32] Specific explanations for these findings are currently unavailable; however, this process seems to mirror early Y-chromosome research in which a clinical effect is detected before its etiology. With advancements in genomic research and technology, we will eventually fill in the knowledge gaps and gain a better understanding of how these genes can influence more than just fertility. This area of research presents the new frontier for Y-chromosome research and will help further elucidate its function.

Y Chromosome Structure

The Y chromosome, once thought to be a defective or nonfunctional chromosome, encodes multiple genes that are necessary for male phenotypic development and reproduction. The basic map of the Y chromosome is composed of adjacent euchromatic and heterochromatic segments flanked by pseudoautosomal regions on either end (Figure 5.1). Structurally, the Y chromosome consists of 59 million DNA base pairs with the MSY accounting for 95% of the chromosome's length. The MSY consists of both euchromatic and heterochromatic portions and is the section of the Y chromosome responsible for male-specific differentiation (Figure 5.1).[9] The euchromatic portion of the MSY is approximately 23 megabases (Mb) in length and consists of three distinct sequence classes: X-transposed, X-degenerate, and ampliconic (Figure 5.2). X-transposed and X-degenerate originate from the X chromosome with transcriptional products expressed throughout the body.[9] Ampliconic sequences are a dense area of transcription where the products are testis specific. On the long arm of the Y chromosome (Yq), the ampliconic sequences consist of eight massive palindromic sequences (P1–P8) that range from 9 kilobases (kb) to 1.45 Mb each. These palindromic sequences express a 99.97% intrapalindromic identity and contribute to unique form of replication that takes place in the Y chromosome.[33]

In meiosis, somatic chromosomes replicate and exchange DNA sequences through recombination. The lack of a homologous chromosome means that the Y chromosome is unable proceed with recombination and must undergo a process called gene conversion to exchange and maintain genetic material.[9] Gene conversion is the nonreciprocal transfer of genetic information between homologous sequences. In paired chromosomes this process is utilized to repair DNA. The process begins with recognition of a base pair

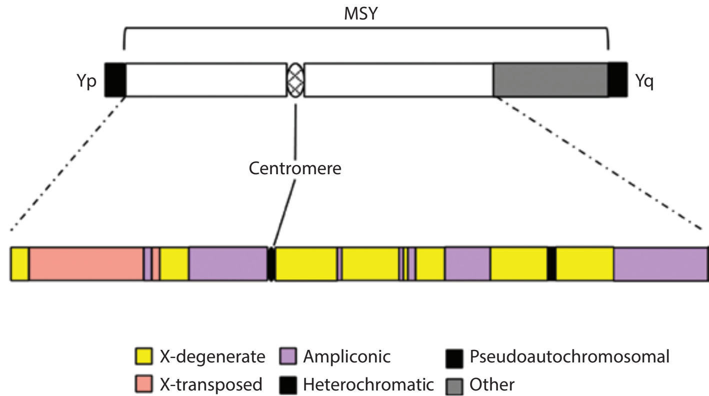

FIGURE 5.2 Euchromatic map of the Y chromosome.

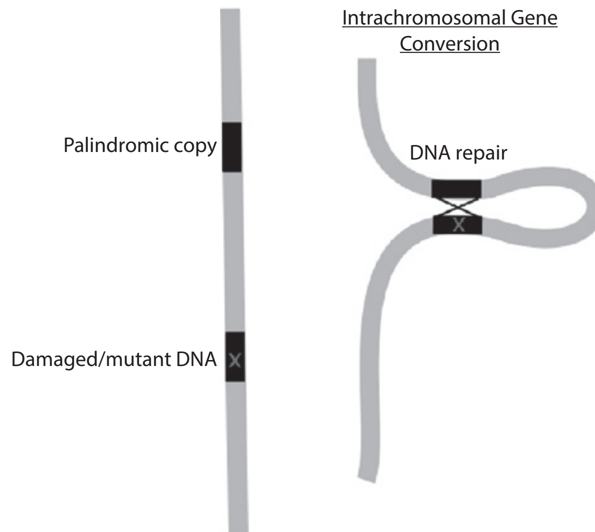

FIGURE 5.3 Intrachromosomal gene conversion for DNA repair.

mismatch or double-strand DNA break. A secondary allele on the homologous chromosome acts as a template and donates its sequence to repair the broken DNA and two identical copies are formed.

Instead of pairing with a homologous chromosome, the Y chromosome uses the high sequence homology of its palindromic sequences to undergo gene conversion (Figure 5.3). The strong sequence similarity between Y-chromosome palindromes allows for allelic recognition and maintenance of DNA sequence fidelity. This process is advantageous for the Y chromosome. The singular nature of the Y chromosome requires that back-up gene copies reside within the chromosome itself and the ampliconic sequences ensure that the genes contained with them are not lost.

Y-Chromosome Microdeletions

This high level of sequence identity within palindromes can also cause improper allelic alignment. Nonallelic or ectopic gene conversion occurs when nonhomologous sequences erroneously combine and intervening genetic material can be deleted (Figure 5.4). These deletions are called microdeletions because they are detected on light microscopic karyotype analysis. Vogt et al. was one of the first to describe Y-chromosome microdeletions when they described common deletion patterns within a section of Yq in azoospermic and

severe oligozoospermic patients and named this specific locus the azoospermic factor or AZF.[11] Within the AZF they described three nonoverlapping subregions that exhibited different phases of spermatogenesis arrest and designated them AZFa, AZFb, and AZFc[11] (Figures 5.5 and 5.6; Table 5.2).

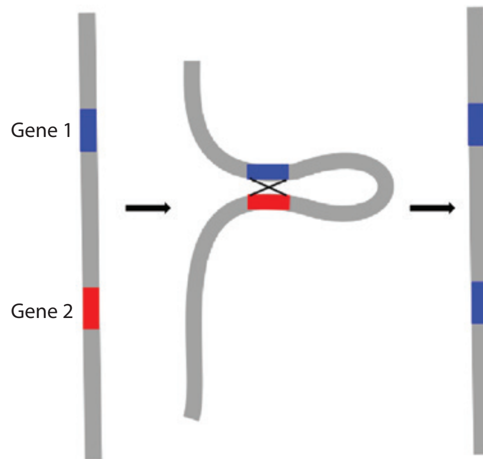

FIGURE 5.4 Erroneous, nonhomologous gene conversion resulting in YCMD.

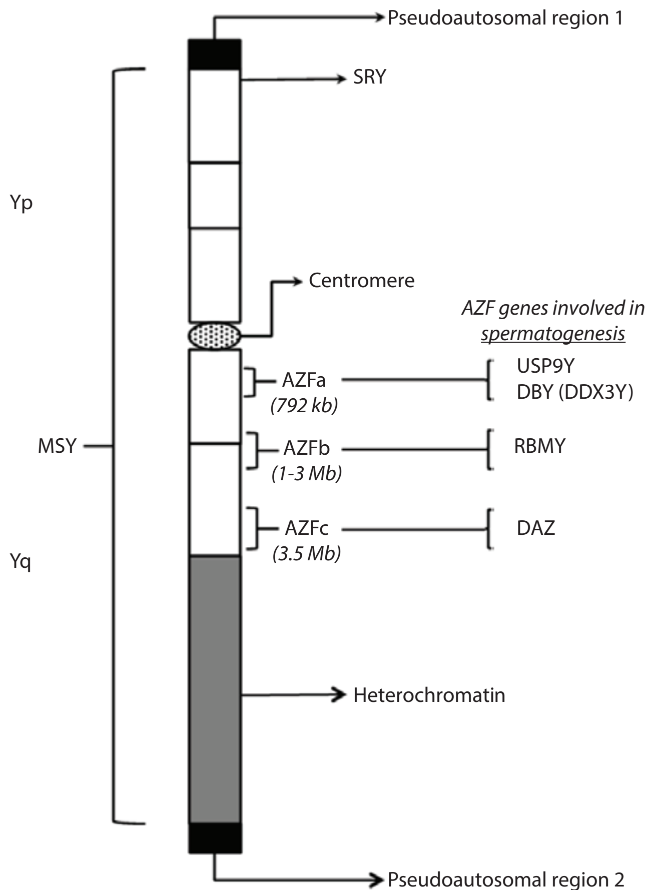

FIGURE 5.5 Y-chromosome map with AZF deletions and associated genes.

TABLE 5.2

Historical Review of the Initial Studies on AZ

Study	Year	Findings
Tiepolo et al.[17]	1976	YCMD first described using light microscopy and establishment of the AZF
Vogt et al.[87]	1992	Discovery of AZFa and AZFb as YCMD
Ma et al.[38]	1993	First study to describe RBMY (then YRRM) as an AZF candidate gene
Reijo et al.[44]	1995	First study to describe DAZ gene as an AZF candidate gene
Vogt et al.[11]	1996	Defined three distinct YCMD patterns within Yq11 and named them AZFa, AZFb, AZFc; each deletion resulted in a different stage of arrest in spermatogenesis
Brown et al.[34]	1998	First study to describe USP9Y (then DFFRY) gene as an AZF candidate gene
Kuroda-Kawaguchi et al.[10]	2001	Entire AZFc region sequenced
Repping et al.[12]	2002	First study to describe the AZFbc region
Repping et al.[51]	2003	First study to describe the AZFc subregion deletion, gr/gr

Abbreviation: YCMD, Y-chromosome microdeletions.

TABLE 5.3

Prevalence, Associated Genes, and Prognostic Information in AZF Deletions

Deletion	Basics	Size	AZF Candidate Genes	Prognosis
AZFa	Rare	1100 kb	USP9Y, DBY	Sertoli cell only, no sperm
AZFb	Rare	6.2 Mb	RBMY	Maturation arrest, no sperm
AZFbc	Rare	7.7 Mb	RBMY, DAZ	No sperm
AZFc	10% of men with NOA, 1:4000 overall	3.5 Mb	DAZ	70% chance of sperm on micro-TESE, rarely sperm in ejaculate
gr/gr	2.4% overall	1.6 Mb	DAZ, reduces copy number	64% chance of sperm on micro-TESE, normal to reduced sperm count in ejaculate

AZFa Deletions

The AZFa region is about 1100 kb in length and contains two genes recognized as vital to spermatogenesis, USP9Y and DDX3Y or DBY (Table 5.3). Ubiquitin-specific protease 9 on the Y chromosome or USP9Y was the first gene identified within AZFa. This gene differs from other AZF candidate genes because it is a single copy gene on the Y chromosome, has an active homologous gene on the X chromosome, and is ubiquitously expressed in a range of tissues.[34,35] One study found that a 4-bp deletion in the exon of the USP9Y gene resulted in a truncated protein and azoospermia.[36] This is unique because most microdeletions are much larger, encompassing multiple genes, thus complicating the reason for the resulting phenotype. This is the first time that a specific mutation was isolated to just one gene and shows that a functional copy of USP9Y on the Y chromosome is required for spermatogenesis. The other gene implicated with azoospermia within the AZFa region is the dead box on the Y or DBY gene. It is more frequently deleted than USP9Y and its deletion leads to severe spermatogenic damage. In its ubiquitous transcripts, DBY has a shorter testis-specific transcript that when deleted may be specific to spermatogenesis failure.[37] Entire AZFa deletion is rare, occurring in only 0.5%–4% of Y-chromosome microdeletions, and histologically results in the complete absence of germ cells or Sertoli cell only syndrome (SCOS) (Table 5.3). This absence of sperm means that testicular extraction of sperm (TESE) for intracytoplasmic insemination (ICSI) is universally not recommended.

AZFb and AZFbc Deletions

AZFb is the largest of the three spanning 6.2 Mb, extending from palindrome 5 to the proximal portion of palindrome 1 (P5/proximal-P1) (Figure 5.6). Deletion patterns within this region can range from deletion of the AZFb or just parts, including flanking regions. The main gene in this region is RBMY1, a testis-specific splicing factor. RBMY1 was one of the first AZF candidate genes to be described.[38] RBMY1 belongs to the RBMY gene family, a family of 20–50 testis-specific genes and pseudogenes spread over both arms of the Y chromosome.[39] Within this family, RBMY1 is the only one that is actively

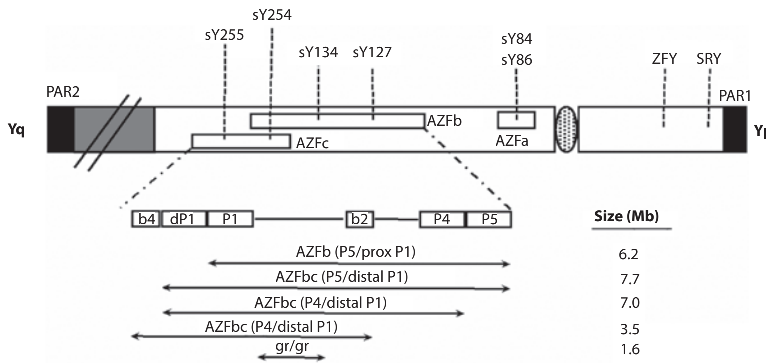

FIGURE 5.6 AZF deletion patterns with STS primers.

transcribed and is concentrated within the AZFb region.[40] Deletion of the RBMY mouse homolog, Rbm, results in infertility.[41] The exact function of RBMY1 in human spermatogenesis is unclear, other than it is a nuclear protein involved in spermatogenetic pre-mRNA splicing.[42] Taken together the testis specificity, AZFb location, and lack of gene expression in YCMD make RBMY1 an ideal AZF gene candidate. Deletion occurs in 1%–5% of cases and causes maturation arrest at the primary spermatocyte stage (Table 5.3). The suspension of the spermatocytes in a premeiotic, polyploidy state restricts their use in assisted reproduction and therefore TESE is not recommended.

Originally AZFa, AZFb, and AZFc were thought to be nonoverlapping regions of the AZF locus. Through advances in molecular genetics, a fourth common deletion pattern was found involving AZFb and AZFc.[12,43] AZFbc has the same contents as AZFb (P5/proximal P1), with the addition of the distal portion of palindrome 1 to form P5/distal P1 (Table 5.3). The deletion encompasses up to 7.7 Mb and results in the removal of 42 genes or transcripts. Despite the large deletion the only phenotype impairment is in spermatogenesis, further highlighting the specialization of this locus. Because the deletion area encompasses the AZFb region, TESE is not recommended.

AZFc Deletions

The AZFc region is 3.5 Mb in length and is located between amplicons b2 and b4 (Figure 5.6). The most recognized gene for spermatogenesis within this region is the DAZ (deleted in azoospermia) gene[44] (Table 5.3). This gene belongs to a multigene family and is clustered within the AZFc region.[45] Additionally, DAZ is testis specific and expressed throughout all stages of spermatogenesis.[46,47] Deletion of AZFc is the most common Y-chromosome microdeletion, accounting for 12% of nonobstructive azoospermia and 6% of oligoozospermia cases.[11,48] The increased incidence of AZFc deletion is the result of a high arm-to-arm sequence identity (99.97%) that allows for more nonhomologous recombination. The phenotypic outcomes of complete AZFc deletions are very favorable with the potential for hypospermatogenesis. Although a majority of these cases are azoospermic, the testicular sperm retrieval rate is significant; therefore these patients are appropriate candidates for micro-TESE and ICSI.[49]

In addition to complete deletions, there is a prevalent subdeletion of AZFc called gr/gr that has been associated with low sperm counts and subfertility[12,50] (Table 5.3). The gr/gr subdeletion was named after the green and red fluorescent probes used for detection, and it consists of approximately 1.6 Mb containing two copies of the DAZ gene.[51] Therefore, the deletion of gr/gr would only diminish rather than eliminate all DAZ copies as in AZFc deletions. Unique to the gr/gr deletion, compared with complete AZF deletions, is its presence in normospermic men and heritability.[50,52] The frequency and spermatic phenotype seem to vary according to geography and ethnicity.[50,52,53] The variable affect of the gr/gr deletion on spermatogenic failure suggests a lower penetrance than complete YCMD.[51] Because identification of a gr/gr does not alter management, it is not part of the standard YCMD testing.

Unlike AZFa and AZFb, patients with the AZFc deletion have a high chance of sperm retrieval, making them appropriate candidates for micro-TESE. Although individual outcomes may vary, AZFc

deletions significantly reduce sperm number without interfering with quality. In fact, sperm are found in up to 70% of AZFc men who undergo micro-TESE.[54] Therefore, clinical management is dependent upon the type of AZF deletion and is the reason microdeletion testing is recommended in patients with severe oligoozospermia or nonobstructive azoospermia.

The Effect of Complete vs. Partial Deletions

Most spermatic phenotypes of AZF deletions are based upon large, overlapping sequence deletions, which result in more severe spermatic phenotypes.[19] As molecular diagnostic techniques improve, so has our ability to detect incomplete or partial deletions. These partial deletions seem to have more favorable clinical outcomes than complete deletions and have been associated with more productive spermatogenesis.[22,55–57] Although smaller deletions are associated with a better prognosis, this is not a universal rule. Depending on the population, subdeletions, such as gr/gr, can be detrimental to spermatogenesis despite their smaller size.[50] Spermatogenic failure within these smaller deletion sequences can help identify regions that are more specific to spermatogenesis. For example, a 4-bp deletion within the USP9Y gene resulted in azoospermia leading to a greater emphasis of its importance in spermatogenesis.[36]

Despite the possible impact, investigation of partial YCMDs is not recommended. Currently, there is not enough evidence that associates a consistent partial deletion pattern with spermatogenesis failure; however, this is subject to change as more information about the Y chromosome becomes available through more sophisticated molecular technologies.

Histological Phenotypes in YCMD

The nature of the YCMDs makes it difficult to prognosticate spermatic histology based on deletion patterns. As mentioned previously there is a theme in specific AZF deletions; however, these outcomes are not universal (Table 5.3). One confounding variable to establishing a reliable prognosis is the intrinsic genetic variability between different populations. Although the similarity of the Y chromosome between individuals is higher than other, much larger, chromosomes, exact gene organization or sequences still exhibit variations and are most apparent between different ethnic groups.[53,56,58–62] Sachdeva et al. compared the European Academy of Andrology (EAA)–recommended STS primers to ones specific to the Indian population and found that EAA recommendations detected only 3% of YCMDs compared to 7.5% with the ethnic-specific primers.[63] Without an identical template for STS deletion detection, we cannot for certain say that a particular deletion pattern will result in specific spermatic histology.

Another problem is the limitation of our technology for Y-chromosome mapping and deletion detection. STS were used to create a Y-chromosome map and are currently used to determine YCMD deletion patterns. The original STS map of the Yq11.23 has been revised several times and its accuracy is still in question.[21,44,64,65] The use of STS is a double-edged sword because not using enough STS primers means that deletions will be missed, whereas using too many results in detection of irrelevant, polymorphisms not related to fertility.[66] Therefore, our ability to determine spermatic phenotypes based on deletion patterns is hindered by our current detection methods.

Clinical Presentation and Assessment

Y-chromosome microdeletion testing begins with an AUA-recommended infertility work-up consisting of a detailed medical and reproductive history, physical examination, and two semen samples[67] (Figure 5.7). It is important to rule out any reversible causes of infertility before undergoing further testing. Patients with YCMD will not present with an obvious etiology for infertility and often their histories will not provide anything substantial. Since most YCMD deletions occur spontaneously, patients will not report a family history of infertility. The only significant finding on physical examination could be small testis size; however, this finding is not unique to YCMD.[68,69]

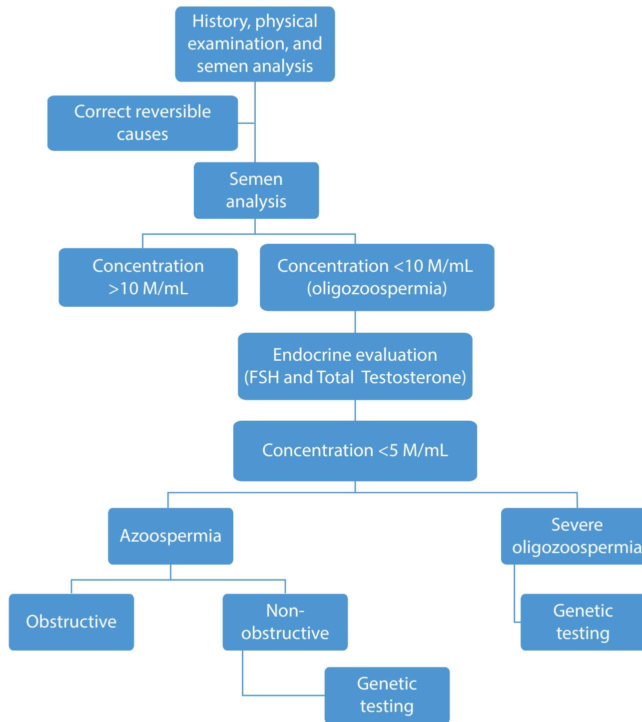

FIGURE 5.7 Clinical algorithm for the assessment of male infertility patients.

Semen analysis is a useful tool in understanding male infertility and is used as an indication for genetic testing. The AUA practice guideline for infertility recommends that genetic testing be ordered in nonobstructive azoospermic or oligozoospermic (sperm count less than 5 million/mL) men.[67] Despite the clinical usefulness of the semen analysis, it is not a direct measure of fertility.[70] Patients with semen values below World Health Organization recommended ranges can conceive without the help of assisted reproductive technologies. On the other hand, men with semen variables within the same reference values may be infertile. The only true independent predictor of fertility potential from a semen sample is azoospermia. Other means to determine more precise male reproductive potential such as adjunctive blood or nonspermatic semen analysis are being explored; however, findings from these tests are not significant enough to impact clinical management.[71–74] Despite these predictive limitations, semen analysis is still regarded as one of the most useful clinical tools in the diagnosis of infertility.

A dysfunctional endocrine system is a well-recognized cause of infertility; however, it is not common and only recommended in men with abnormal semen analysis, impaired sexual function, or other clinical findings suggestive of endocrine disruption. The strongest indication for endocrine evaluation based on semen analysis is a semen concentration of less than 10 million/mL.[13,67] Initial testing includes FSH and total testosterone with repeat total testosterone, free testosterone, prolactin, luteinizing hormone (LH), and thyroid-stimulating hormone (TSH) indicated for low initial testosterone. Since men with YCMD fall into this category of low semen concentration, many will undergo an endocrine evaluation. Men with YCMD on endocrine evaluation may have elevated FSH with low to normal testosterone; however, this finding may not be true in all cases.[58,68] The increase in FSH is the response to a lack of spermatogenesis in YCMDs. This endocrine profile is similar to primary testicular failure, however, with a reduced magnitude.

The next step is to determine whether the azoospermia is considered nonobstructive (NOA) or obstructive azoospermia (OA). OA occurs in about 15%–20% of men with azoospermia and can occur anywhere along the course from the testicle to the ejaculatory ducts. OA is usually detected with a good history and physical examination. Men with OA have a history of genital surgery, infection, or

congenital abnormalities. On physical examination they demonstrate normal testicular volume and indurated epididymis, whereas NOA exhibits small, soft testes with a flat, soft epididymis.[69] Another difference between OA and NOA is the hormonal profile. OA will have normal FSH levels, whereas NOA will have elevated FSH.

Once a diagnosis of NOA is established, genetic causes for infertility can be considered. Genetic screening for infertility consists of screenings for cystic fibrosis gene mutation, karyotype analysis, and YCMD testing, which are ordered at the same time. Even with the help of these genetic tests, the causes of infertility may still be unknown. Currently, all known genetic disorders of infertility only account for 30% of cases.[4] This means that 70% of genetic causes of infertility are still unknown. This high percentage results from the complexity of spermatogenesis. Thousands of genes must coordinate to produce mature sperm and the failure of just one of these genes can disrupt the entire process. Given the sheer number of genes involved and our limited knowledge of the genome, it is possible that a large majority of male infertility is from unknown causes. Although these three tests account for some of the genetics of infertility, they are by no means exhaustive.

YCMD Testing

Diagnostic testing for Y-chromosome microdeletion is performed by PCR assays. STS primers identify various sections related to microdeletions within the AZF locus (Figure 5.6). It is important that STS primers come from regions of the Y chromosome that are conserved within azoospermic/oligozoospermic men with YCMD. STS primers are used to amplify anonymous regions within each AZF region. Microdeletion detection occurs from a lack of amplification, when the STS primers are unable to bind to their deleted complementary DNA. Gene-specific amplification within these regions does not increase the detection rate of clinically relevant microdeletions, emphasizing that the location of the primer is more important than the amplification content.[59]

Determining the appropriate number of the STS primers for detection is important. Using not enough primers decreases screening sensitivity, missing important deletions. A high number of primers can improve accuracy; however, they can also produce clinically irrelevant information. Simoni et al. found that the frequency of YCMDs is independent of the number of STS primers used.[75] Guidelines addressing the appropriate number of STS primers vary. The AUA Guidelines have no minimum recommendation for the number of primers used, whereas the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQM) suggest a minimum of eight primers: two for each AZF region, one for SRY, and one control (ZFY/ZFX gene)[7,67] (Figure 5.6). This European recommendation is the first attempt to standardize the number of primers and enables the detection of over 95% of deletions within three AZF regions.[7]

Because of the high sequence identity and multiple DNA primer locations within the AZF locus, the EAA/EMQM recommend multiplex PCR assays.[7] Standard PCR assays amplify only one sequence at a time, whereas multiplex PCR assays can amplify multiple sequences simultaneously. This allows for more efficient and cost-effective detection of YCMDs. The multiplex PCR occurs in two stages. The first stage is deletion detection where samples are compared with fertile male, female, and the patients, ZFY/ZFX gene. Once a deletion is detected, the next stage uses another set of primers to determine the extent of the deletion. Compared with previous genomic screening technologies, multiplex PCR provides a safe and effective way to detect clinically significant YCMDs.

IVF Success and Transgenerational Impacts of YCMD

Men with AZFc YCMDs and successful sperm retrieval are appropriate candidates for in vitro fertilization (IVF). The immotility of the sperm collected makes intracytoplasmic sperm injection or ICSI the treatment of choice. Despite the collection of mature sperm, fertilization and embryogenesis outcomes may vary. Two studies found that patients with AZFc microdeletions had lower fertilization rates when compared with azoospermic/oligozoospermic men without YCMDs.[76,77] Additionally, YCMD has also been

TABLE 5.4

ICSI Outcomes in Patients with YCMD

Study	Year	Cohort	Control	Findings
Zhu et al.[76]	2015	61 infertile men with AZFc deletion undergoing ICSI	ICSI in azo/oligo controls without YCMD	AZFc deletion cohorts had lower fertilization rate No difference in available or transferred embryos clinical pregnancy rate, implantation rate, miscarriage rate, preterm rate
Liu et al.[79]	2013	123 oligozoospermic patients with AZFc deletion undergoing ICSI	ICSI in azo/oligo controls without YCMD	No differences in transferred embryos, good embryo rates, implantation rates, biochemical and clinical pregnancy rates, ectopic pregnancy rates, miscarriage rates, preterm rates
Van Golde et al.[77]	2001	8 couples with AZFc deletion undergoing ICSI	ICSI in patients with sperm in the ejaculate	AZFc deletion cohort lower fertilization rate poorer embryo quality No differences in pregnancy rate, implantation rate Take-home baby rates
Mateu et al.[78]	2010	Six patients with AZFc microdeletions	ICSI in azo/severe oligo without YCMD	AZFc deletion cohort More embryonic chromosomal aneuploidies More monosomy X embryos No differences in fertilization rate, implantation rate, pregnancy rate, miscarriage rate

associated with poor embryo quality and a higher incidence of chromosomal aneuploidies; however, these limitations did not translate into different pregnancy or birth rates[77,78] (Table 5.4).

Fertilization and embryogenesis data are far from conclusive. In one of the largest studies of ICSI outcomes in YCMD patients, Liu et al. found no differences in the number of transferred embryos, good embryo rates, implantation rates, or pregnancy rates.[79] Regardless of the difference in fertilization rate and embryo quality, pregnancy outcomes were similar to couples without microdeletions undergoing ICSI with no significant difference in pregnancy rate, ectopic pregnancies, miscarriages, preterm birth rates, and number of birth defects.[76–79] Taken together these findings suggest that ICSI is a successful IVF procedure in men with YCMD.

Before assisted reproductive technologies, defective or absent genes from infertile men would not be passed on to their progeny; however, as more couples seek infertility treatment, the consequences of bypassing this Darwinian “survival of the fittest” step will become more prevalent. The Y chromosome is exclusive to the male lineage and, because it does not undergo genetic recombination, its information is well conserved across generations. Most YCMDs occur spontaneously and after they occur would be passed on from father to male offspring through sex-linked inheritance. Studies investigating these inheritance patterns of men with AZFc deletions undergoing ICSI found that most male offspring had some form of Y-chromosome microdeletion consisting of an identical, expanded, or de novo deletion pattern.[80,81] However, this result is not conclusive. A study by Liu et al. assessed YCMD inheritance in 228 sons from fathers with YCMD.[82] They looked at 19 candidate genes within the AZF region and found no significant difference in the detection rate of the father’s YCMD group. Although this is the only study that disproves Y-chromosomal inheritance pattern of these deletions, the large sample size means that the inheritance pattern is more complicated than previously thought.

The inevitable heritability of Y-chromosome microdeletions from ICSI necessitates that these couples seek genetic counseling to understand the risk to their male offspring. Although most couples choose to proceed with ICSI, preimplantation genetic diagnosis for female sex selection, sperm donation, and adoption need to be discussed as viable options.[83]

Future of YCMD

The future of YCMD screening is closely associated with the accuracy and cost of genetic testing. Genetic causes of male infertility have been studied using a wide area of genetic targets such as microarray studies, proteomics, metabolomics, and genomics; however, none of these has significantly altered the current

treatment algorithm (Figure 5.7). This deficiency in novel screening tests represents the complexity and our limitations in defining the role of the Y chromosome. Further complicating our understanding is the lack of reliable animal models for spermatogenesis and the genetic differences between infertile male cohorts.[2,53,58–62]

Our knowledge is limited by the accuracy and reliability of our current genetic tests. The difficulty with assessment of YCMDs is the identification of a single target gene. Many of the genes within the AZF implicated in spermatogenesis have multiple copies and determining which single or combination of active genes is critical to developing more specific screening tests. Identification of these target genes will help isolate clinically relevant deletions, providing better prognostic information.

Another factor that will influence the future of YCMD screening is cost. In general, costs of genetic screening tests have continued to diminish. The cost of sequencing the first human genome in 2003 was $2.7 billion, compared with the current cost of just over $1000.[84] This cost reduction is from improvements in computer processing. Moore's law states that the computer processing power will double every 2 years, making more powerful computers cheaper with time. This reduction in computational cost, along with the increased use of computers in molecular studies, will make genome sequencing more common in clinical practice, allowing for earlier identification of genetic abnormalities. In the future, genome sequencing may eventually become part of the initial doctor's visit.

Conclusion

Most Y-chromosome microdeletions occur spontaneously as a result of incorrect nonhomologous recombination and are considered part of male infertility testing in men with nonobstructive azoospermia or severe oligozoospermia. Exact deletion identification is important because specific microdeletion sequences have prognostic value resulting in different clinical management. Apart from guiding clinical decision making, these tests can help manage fertility expectations of couples seeking help. Prior to pursuing genetic infertility testing, all patients must be informed of the possible outcomes and how these results can change their fertility options as well as impact their future offspring.

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6

The Application of Genetic Tests in an Assisted Reproduction Unit: Sperm FISH

Lorena Rodrigo and Carmen Rubio

The Scientific and Biological Bases of Sperm FISH

Intracytoplasmic sperm injection (ICSI) allows overcoming the natural barrier offered by the oocyte to sperm fertilization in couples with severe male factor infertility. However, in prenatal diagnosis from ICSI pregnancies, an increased incidence of de novo sex chromosome aneuploidies and structural rearrangements has been observed.[1,2] Most of them are of paternal origin[3,4] and are mostly attributed to the sperm quality of the infertile men.[5,6] Between 2% and 26% of the infertile men with normal karyotype show cytogenetic anomalies confined to the germ cell line,[7,8] which makes sperm chromosome studies particularly interesting.

Sperm fluorescence in situ hybridization (FISH) uses fluorescence DNA probes directed to specific DNA sequences in the interphase sperm nuclei. Visualizing the hybridization signals using fluorescence microscopy, it is possible to identify numerical chromosome abnormalities in the nucleus of ejaculated, epididymal, and testicular sperm. The simultaneous use of multiple probes specific for the different chromosomes allows rapid and relatively simple evaluation of a large number of sperm, enabling the detection of some structural and numerical abnormalities.[9,10]

Sperm FISH analyses corroborate previous reports with altered meiosis in infertile men, as they have shown higher aneuploidy rates for chromosome 21 and sex chromosomes due to meiotic nondisjunction.[11–13] That is why sex chromosomes and chromosome 21 together with chromosomes 13 and 18 are most commonly included in these studies. Moreover, numerical abnormalities for these chromosomes can lead to potentially viable abnormal pregnancies, including Patau, Edwards, Down, Turner, Klinefelter, 47,XXX, and 47,XYY syndromes. Thereby, sperm FISH allows the evaluation of paternal risk for the transmission of chromosome abnormalities to the offspring.

Analytical Techniques in Order to Measure Sperm FISH

Due to the nature of the spermatozoa, FISH protocol for sperm analysis requires the following steps, which are also summarized in Figure 6.1.

Sperm Fixation

Previous to the hybridization, spermatozoa must be fixed maintaining their morphology and allowing permeability to the DNA probes. After centrifugation with sperm washing media, the supernatant containing the seminal plasma is discarded and the pellet with the spermatozoa is fixed using Carnoy solution (methanol/glacial acetic acid = 3:1). The fixed spermatozoa are spread on glass slides avoiding overlapping.

Nucleus Decondensation

Sperm heads have a tightly compacted nucleus due to the presence of disulfide bridges between protamines; this condensation of nuclear chromatin makes it inaccessible to DNA probes. To solve this

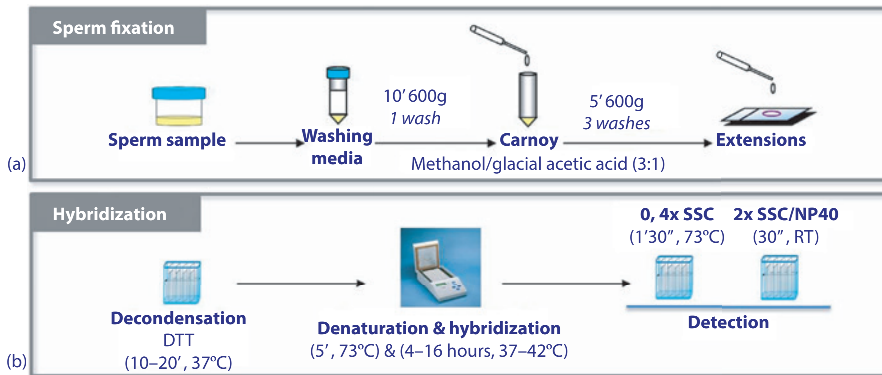

FIGURE 6.1 The steps in (a) sperm fixation and (b) hybridization.

problem, a pretreatment is performed by incubation with reducing agents and dehydration with ethanol. Reducing agents (e.g., dithiothreitol [DTT]) break the disulfide bonds and produce nuclear chromatin decondensation allowing subsequent hybridization with DNA probes.

Denaturation and Hybridization

Double-strand DNA denaturation of the sperm and FISH probes is carried out after incubation at high temperature (70°C–74°C). After denaturation, both DNAs are coincubated and hybridized to form a duplex of complementary strands. Hybridization protocols vary according to the type of FISH probe used, requiring different times and temperatures of hybridization (commonly between 4 and 16 hours at 37°C–42°C).

FISH analysis on sperm is commonly performed using centromeric, locus-specific, and subtelomeric fluorescent DNA probes. For segregation studies in structural rearrangements, specific combinations of these three types of probes are designed for each specific rearrangement. However, in carriers of numerical sex chromosome abnormalities and also in normal karyotype infertile men, the most widely analyzed are chromosomes 13, 18, 21, X, and Y using centromeric and locus-specific probes.

Detection

Excess DNA probes hybridized to unspecific complementary sequences are removed by astringent washes at high temperatures and low saline concentrations (e.g., with saline sodium citrate [SSC]). Finally, a counterstain is applied to allow the visualization of the sperm nucleus (e.g., 4´,6-diamino-2-fenilindol [DAPI] or DAPI/Antifade).

Signal Visualization and Evaluation

The hybridization signals are visualized using a fluorescent microscope equipped with specific filters for each fluorochrome. The evaluation is performed by counting the number of signals for each fluorochrome present in the nucleus of each spermatozoon. The spermatozoa are haploid cells containing one copy of each autosome and one sex chromosome, X bearing or Y bearing. After the evaluation of the fluorescent signals using the criteria described by Blanco et al.,[14] the spermatozoa can be classified (see Figure 6.2) as follows:

- *Haploid normal:* when it shows one signal for each of the autosomes evaluated, and one signal for the sex chromosomes (X or Y)
- *Disomic:* when it shows two signals for one of the chromosomes evaluated, and one signal for the remaining chromosomes evaluated
- *Diploid:* when it shows two signals for each of the chromosomes evaluated

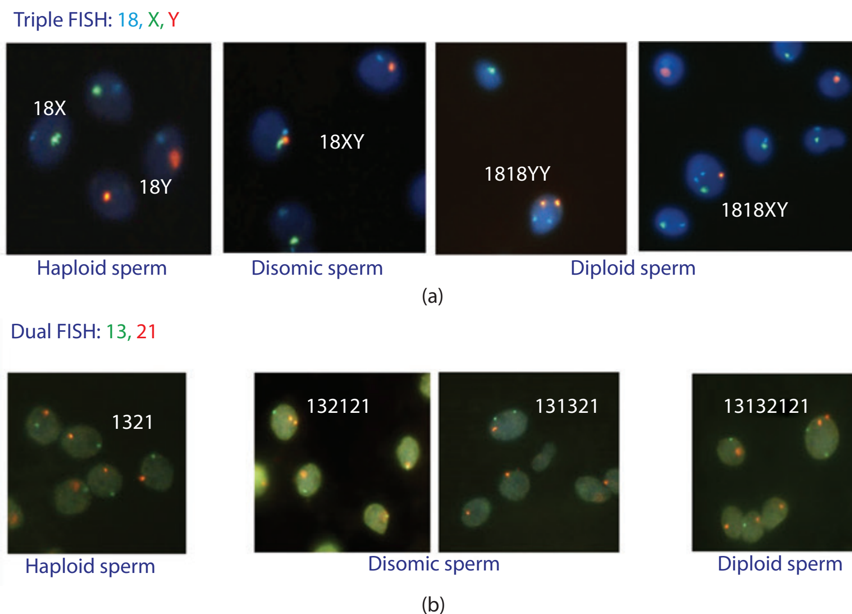

FIGURE 6.2 (a) Triple FISH 18, X, Y and (b) dual FISH 13, 21.

It is recommended to score only spermatozoa with clear hybridization signals, avoiding the analysis of cells with missing signals, as they could represent either nullisomies or hybridization failures.

After evaluation, a sperm sample is classified as abnormal when a significant increase in abnormal sperm (disomies and/or diploidies) is observed compared to the incidence observed in a control population of normozoospermic fertile men.

The total aneuploidy rate in normozoospermic men has been estimated to be 6%, with 0.12% mean disomy for autosomes and 0.31% mean disomy for the sex chromosomes.[10] Due to this low aneuploidy rate, for clinical applications a minimal number of 1000 sperm per sample should be scored; nevertheless, this number may be limited in cases of low sperm count such as cryptozoospermic and azoospermic men.

Clinical Evidence

At the *clinical level*, an increase in spermatozoa with chromosomal abnormalities has been associated with a decrease in pregnancy rates and higher miscarriage risk in infertile couples undergoing ICSI cycles. Rubio et al.[15] compared the outcome of 108 ICSI cycles from patients with a normal sperm FISH result with that of 23 ICSI cycles from patients with an abnormal sperm FISH result, resulting in similar fertilization rates (71.5% vs. 74.5%), higher pregnancy rate (36.5% vs. 23.6%), and lower miscarriage rate (54.8% vs. 80.0%) in the group of patients with normal FISH result. Similarly, Burrello et al.[16] analyzed the clinical results in a series of 48 patients undergoing ICSI. They observed lower pregnancy (34% vs. 75%) and implantation (13% vs. 34%) rates, and higher miscarriage rate (38.9% vs. 11.1%) in patients with higher incidence of aneuploid sperm compared to patients with normal incidence (considering as normal below 1.55%). However, fertilization rate and embryo quality were similar in both cases. Nicopoullos et al.[17] also found significantly higher sperm aneuploidy rate in patients who did not achieve pregnancy compared to patients who achieved pregnancy after ICSI (2.37% vs. 1.18%). Moreover, the probability of achieving a clinical pregnancy decreased by 2.6 times for every 1% increase in the sperm aneuploidy rate. In another study, Petit et al.[18] found higher rates of aneuploidy and diploidy in sperm of men who did not conceive after ≥4 ICSI cycles compared with those who got pregnant after one to three ICSI cycles.

At the *embryo level*, preimplantation genetic screening (PGS) has allowed the evaluation of the impact of higher rates of sperm numerical chromosome abnormalities on the chromosomal constitution of embryos. Several reports described higher rates of abnormal embryos, noting a high incidence of aneuploidy for sex chromosomes and mosaic embryos.[19–25] Patients with severe oligozoospermia or cryptozoospermia that were carriers of Y-chromosome microdeletions showed higher increase of aneuploid embryos, highlighting monosomy X.[26] More particularly, different effects on embryo chromosome constitution have been described according to the type of sperm chromosomal abnormality detected. An increase in the percentage of spermatozoa with sex chromosome disomies has been associated with an increase of embryo aneuploidies compatible with life (Patau, Edwards, Down, Klinefelter, and Turner syndromes, and trisomies XXX and XYY), whereas an increase in diploid spermatozoa has been related to an increase in triploid embryos, which mostly miscarry before delivery.[24]

At the *offspring level*, several studies performed in parents of children with Down, Kinefelter, and Turner syndromes have shown increase in sperm chromosomal abnormalities associated with the chromosomopathies observed in the children. Thus, in a study conducted on two fathers of children with Down syndrome of paternal origin, the sperm disomy 21 rates were reported as 0.75% and 0.78%.[27] Similar studies in couples with miscarriages or children with sex chromosome abnormalities (Turner or Klinefelter syndrome) have reported high incidences of sperm aneuploidy for sex chromosomes, ranging from 0.20% to 24.7%.[28–33]

Clinical Use

At present, sperm FISH technique has become one of the main tools used for the diagnosis of male infertility and the evaluation of paternal risk for the transmission of chromosome abnormalities to the offspring. Indications to perform sperm FISH test are listed as follows:

1. Normal karyotype patients with
   a. Impaired sperm parameters: oligozoospermia and severe teratozoospermia
   b. Nonobstructive azoospermia
   c. Impaired meiosis in testicular analysis
   d. Chemotherapy and radiotherapy treatments
   e. Clinical history of unknown recurrent miscarriage
   f. Clinical history of repetitive implantation failure
   g. Previous pregnancy with chromosomopathy
2. Abnormal karyotype patients with
   a. Numerical abnormalities for sex chromosomes: Klinefelter syndrome (XXY) and XYY men
   b. Structural chromosome abnormalities: reciprocal and Robertsonian translocations, and inversions

Results

Sperm FISH in Normal Karyotype Men

FISH analysis on sperm has identified normal karyotype men with higher risk of sperm chromosome abnormalities, such as men undergoing chemotherapy or radiotherapy and men with chromosomally abnormal offspring of paternal origin. In the first group, a fivefold increase of diploid sperm and sperm with aneuploidies for autosomes and gonosomes after the treatment has been observed compared to their basal level.[34,35] In the second group, the fathers of the affected children have shown incidences of 1%–20% of aneuploid sperm affecting the chromosomes of the alteration.[27,29,32,33]

Most publications about infertile men have shown higher incidence of sperm aneuploidies compared to fertile population.[36–43] In this case, FISH analysis has been applied mainly to patients with impaired sperm parameters (oligozoospermia, severe teratozoospermia, and azoospermia) and to couples with clinical history of recurrent miscarriage or repetitive implantation failure.[15,44–49]

Sperm FISH in Carriers of Chromosome Abnormalities

About 5.8% oligozoospermic or azoospermic men are carriers of numerical or structural chromosome abnormalities affecting the spermatogenesis.[50] Infertile men with Klinefelter or 47,XYY syndrome are at risk of low sperm production with poor sperm quality and abnormal chromosome constitution.[24,51–53] In these men, incidences of 1%–20% of spermatozoa with aneuploidies for the sex chromosomes and 1% of diploid sperm have been described.[54]

Carriers of balanced chromosomal rearrangements such as Robertsonian or reciprocal translocations and inversions course with a variable range of alterations in their gametogenesis, resulting in normozoospermia, oligozoospermia, or even azoospermia. After spermatogenesis, the spermatozoa can also be chromosomally unbalanced in a variable range.[55] The incidences of unbalanced sperm for the chromosomes of the rearrangement are 10%–40% in Robertsonian translocations, 50%–65% in reciprocal translocations, and 1%–55% in inversion carriers.[56]

Methods to Improve the Results

Most published studies related to sperm aneuploidy on infertile men have shown that couples in which men have increased frequencies of numerical sperm chromosomal abnormalities have lower pregnancy and implantation rates and higher miscarriage rate after in vitro fertilization (IVF)/ICSI than other infertile couples. Several groups have proposed PGS as an alternative to improve the possibility of healthy pregnancies in couples with male infertility.[20–22]

The percentage of abnormal embryos ranged between 43% and 78% in patients with oligozoospermia and azoospermia in which an abnormal sperm FISH or an impaired meiosis was reported.[20–24,57–60] Rodrigo et al.[61] retrospectively analyzed the reproductive outcome of male factor infertility couples without history of recurrent miscarriage or implantation failure, who carried out sperm FISH analysis for chromosomes 13, 18, 21, X, and Y. IVF/ICSI cycles without embryo chromosomal analysis in couples with abnormal sperm FISH results showed significantly lower embryo transfer rates (64.0% vs. 84.8%), higher mean number of transferred embryos (2.3 ± 0.9 vs. 2.0 ± 0.6), lower pregnancy rates (22.9% vs. 30.8%), and lower implantation rates (12.4% vs. 21.4%) than patients with normal sperm FISH result. However, PGS cycles in patients with abnormal sperm FISH result had higher pregnancy (39.7% vs. 28.3%) and implantation rates (33.8% vs. 21.4%) than patients with normal sperm FISH result, despite lower mean number of embryos transferred (1.6 ± 0.6 vs. 1.7 ± 0.6). Interestingly, patients with normal sperm FISH results had similar clinical results regardless of IVF/ICSI or PGS; however, patients with abnormal sperm FISH result showed better pregnancy and implantation rates with PGS.

Aneuploidy screening of the 24 chromosomes in couples with male factor infertility offers even better clinical results, with 83.6% cycles having at least one euploid embryo to transfer, resulting in a pregnancy rate per transfer of 62.9%, an implantation rate of 54.2%, and a take-home baby rate of 50.9%.[60]

Test Availability

Commercial sperm FISH kits are not available in the market to do the test at home. However, it is possible to send the sperm samples to genetic reference centers, easily identified via the Web by using key words such as “sperm aneuploidy test” or “sperm FISH test.”

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7

The Application of Genetic Tests in an Assisted Reproduction Unit: Cystic Fibrosis Carrier Screening

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Introduction

Cystic fibrosis (CF) is an autosomal genetic recessive disease that mostly affects the Caucasian population with an incidence estimation of 1 case every 2500–3000 newly live births. The disease is clinically manifested through a high concentration of electrolytes in sweat, pulmonary and gastrointestinal obstruction, pancreatic insufficiency, and infertility in both sexes.

However, the severity of the symptoms can vary considerably. Therefore, there are a number of diseases that cause male infertility and are associated with CF, sharing some symptoms with the classical CF such as congenital bilateral absence of the vas deferens (CBAVD) or congenital unilateral absence of the vas deferens and obstructive azoospermia.

In 1989, the responsible gene for CF,[1] named cystic fibrosis transmembrane conductance regulator (CFTR), was identified. This gene is located on the long arm of chromosome 7 and encodes for a transport protein of the ATP-binding cassette (ABC) family. The protein function is to act as an anionic channel regulated by adenosine cyclic monophosphate (cAMP) allowing the passage of ions Cl^- and HCO^-.[2] In addition, the CFTR channel acts as a regulator of other membrane channels such as the epithelial sodium channel (ENaC), different chlorine channels, and aquaporins (AQPs).[3,4]

Approximately 98% men with CF are infertile and present a CBAVD and thus, an obstructive azoospermia.[2,5] Numerous clinical studies have shown that mutations in the *CFTR* gene are responsible for these diseases. CFTR gene mutations have been identified in patients with CBAVD and, thence, a common CF genetic origin is postulated. It has been proposed that the CBAVD could be a genital primary clinical manifestation of the CF.[5-7]

In addition, mutations in the *CFTR* gene have been found in patients with nonobstructive azoospermia, oligospermia, asthenospermia, and teratospermia,[8,9] which suggests that CFTR may be involved in different fundamental processes in male fertility, such as spermatogenesis or sperm capacitation.

This chapter aims to summarize recently obtained findings that associate male infertility with mutations of the *CFTR* gene, responsible for the CF disease. This chapter also presents a description of the possible role of the CFTR protein in different cell signaling pathways involved in important reproductive processes in male fertility, as well as in the clinical application of the following findings.

Male Infertility and *CFTR* Gene

Male infertility can have different etiologies: alterations in sex chromosomes and autosomes, microdeletions in chromosome regions containing families of genes that regulate spermatogenesis, the phenotypic association observed between CF and BAVD (absence of the vas deferens), among others.[7,10]

CBAVD is anatomically characterized by bilateral absence of vas deferens, atrophy or absence of the seminal vesicle, and atrophy or absence of a portion of the epididymis, which leads to an obstructive azoospermia.[2] Numerous clinical studies have shown that mutations in the *CFTR* gene are responsible for this phenotype.[5,7,11] Almost 100% of patients with CF presented CBAVD,[5] and high-frequency

TABLE 7.1

Classification of CFTR Gene Mutations

Class	Description	Protein	Phenotype	Example
I	Altering production	Total or partial absence of the protein	Severe	G542X
II	Altering maturation	Fail the correct CFTR cellular location	Severe	F508del
III	Altering regulation	Cannot be activated by ATP or cAMP	Severe	G551D
IV	Altering conduction	Reduced rate of Cl^- transport	Mild	R117H
V	Altering messenger RNA stability	Reduced levels of protein	Mild or monosymptomatic	IVS8(T)5
VI	Altering protein stability	Mutations that affect regulations of other channels	Severe	G5551D

heterogeneous mutations in the *CFTR* gene have been observed in patients with CBAVD and without clinical features of CF.[5,9,11]

CFTR gene mutations can be classified into five different classes depending on the functional effects of the protein.[2,4,5,10] Mutations of classes I, II, and III are characterized by the complete loss of functionality of the protein, and they produce a severe phenotype, whereas mutations of classes IV and V exhibit a mild phenotype because they maintain a residual function. As a result of the existence of severe and mild mutations and their combination, the phenotypic heterogeneity observed in patients with CF is explained (Table 7.1).

Also, clinical studies have shown that most patients with BAVD have two different mutant alleles of the *CFTR* gene, one of them being of mild character.[5] This could explain the disparity in the aggressiveness of the phenotype between CF and BAVD. Therefore, the total amount of protein expression depending on the type of mutation could establish the difference between expressing CF or CBAVD.[12]

Similarly, mutations in the *CFTR* gene have been identified in patients with other types of male infertility, such as nonobstructive azoospermia, oligospermia, asthenospermia, and teratospermia.[9,13,14] This finding indicates an association between CFTR gene expression and sperm quality.[15] In addition, the presence of the CFTR protein in Sertoli cells and epithelial cells in rat epididymis and its expression in sperm cells at different stages of spermatogenesis have been identified.[15,16] Therefore, observations of different clinical studies propose that mutations in the *CFTR* gene could be affecting sperm production and maturation and its fertilization ability.

CFTR and Its Role in Spermatogenesis

Spermatogenesis is a complex process whereby, on the one hand, totipotent stem cells or spermatogonia divide to self-renew through mitosis. On the other hand, spermatogonia are divided to produce daughter cells through meiosis. Subsequently, daughter cells will enter into a process of cellular differentiation where they will become spermatocytes. This process occurs in seminiferous microtubules where Sertoli cells are located. These cells are responsible for providing the proper endo- and paracrine environment as well as the structural support necessary for spermatogenesis to be carried out correctly.

Sertoli cells are the only somatic cells that can be found in seminiferous microtubules, and they are responsible for maintaining the blood–testis barrier and secreting electrolytes and testicular fluid to create an optimal environment necessary for the development and maturation of stem cells.[8,17] In addition, these cells contain receptors for testosterone and the follicle-stimulating hormone (FSH) involved as major regulators of spermatogenesis.

The observation of the presence of the CFTR protein in Sertoli cells gave rise to the approach that the channel could have an important action in spermatogenesis and its regulation.[18,19]

Different studies have been conducted to explain the possible role of the CFTR channel in spermatogenesis and the mechanisms through which it would be carried out. Taking into account that Sertoli cells carry out ion and seminiferous fluid secretion, the possible role of the CFTR channel in this function was studied. Finally, no positive results were found, and therefore, it was concluded that the CFTR channel was not involved in these processes.[8]

As mentioned previously, Sertoli cells are responsible for feeding testicular stem cells with the secretion of several proteins and growth factors. These processes are regulated by hormones such as the FSH, which binds to its receptors on Sertoli cells and, as a result, there is an activation of the membrane-bound adenylate cyclase (mAC). mAC generates cAMP from adenosine triphosphate (ATP) that will activate protein kinase A (PKA). This protein will activate a signal transduction cascade and, as a consequence, it will activate factors of transcription such as the cAMP-response element binding protein (CREB).[8,20] CREB is a transcription factor that acts as the main controller of various stages of spermatogenesis. Once CREB is activated, it binds to the cAMP-response element (CRE) in DNA and the recruitment of the transcription machinery occurs. As a result, the transcription of a series of genes involved in the process of spermatogenesis is initiated.[20,21] Subsequently, a new type of adenylate cyclase (AC) was identified in Sertoli cells, called soluble adenylate cyclase (sAC), which is sensitive to HCO^- and Ca^{2+}.[8,21] Thus, the participation of the CFTR channel in the entrance of HCO^- in the Sertoli cell could be a mechanism by means of which the activation of sAC and cAMP/PKA/CREB occurs. This mechanism is essential for spermatogenesis. (Figure 7.1).

FSH plays an essential role in spermatogenesis regulation via cAMP. However, various studies have shown that the full activation of this pathway depends on the CFTR channel because it allows the entrance of HCO^-, and consequently, the activation of sAC. In this way, it is emphasized that the CFTR channel is important in the process of spermatogenesis and mutations or aberrant expressions of CFTR result in an insufficient activation of the CREB pathway, and consequently, there is a decrease in spermatogenesis or azoospermia.[21] This finding explains the association between the presence of mutations in the *CFTR* gene and patients with nonobstructive azoospermia and oligospermia.

Furthermore, Sertoli cells are responsible for the constitution and maintenance of the blood–testis barrier. Adjacent Sertoli cells are held together through tight junctions, and thus, they prevent the exchange of substances between blood and luminal liquid of seminiferous tubules, generating the necessary microenvironment for spermatogonial differentiation.

Several studies have observed that the alteration of the blood–testis barrier through the cessation or removal of tight junctions that are formed causes a failure in spermatogenesis.[8,22] At the same time, scientific evidence of the participation of the CFTR channel in the regulation of the union complexes has been accumulated.[8] In a recent study,[23] it has been discovered that the CFTR channel regulates the activation of the nuclear factor kappa beta (NF-κβ) transcription factor negatively and hence decreases the production of prostaglandin E2 (PGE2), which causes the alteration of tight junctions in different cell types.[8] Accordingly, CFTR could execute a key role in the maintenance of the blood–testis barrier

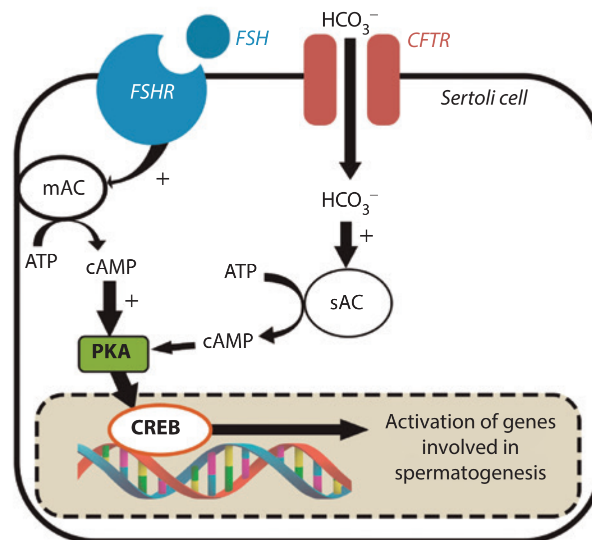

FIGURE 7.1 Signaling pathways mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) channel and follicle-stimulating hormone (FSH) in Sertoli cells.

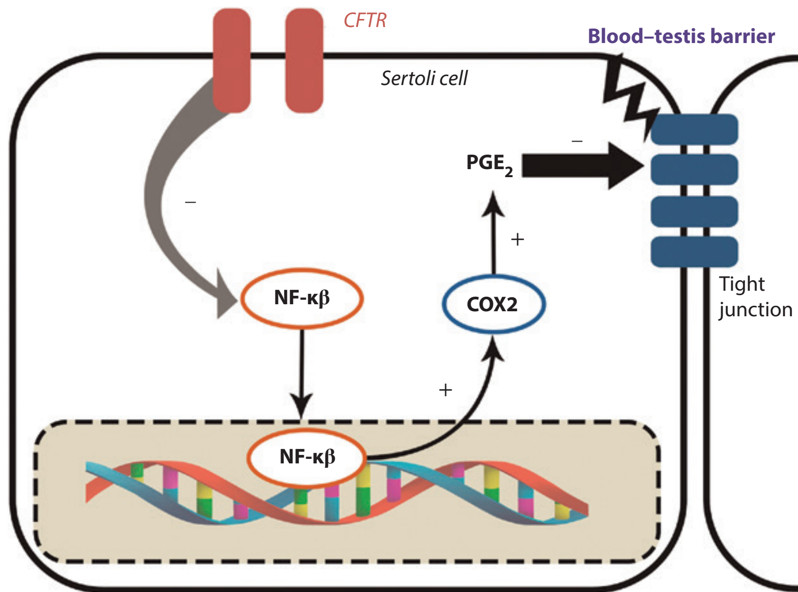

FIGURE 7.2 Signaling pathway mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) channel for maintaining the blood–testis barrier between Sertoli cells.

through the inhibition of PGE2 production (Figure 7.2). Likewise, defects or mutations in the CFTR channel could be causing an alteration in spermatogenesis due to a possible increase in the production of PGE2 and thus the destruction of the blood–testis barrier.

Moreover, further research found an association between infertility caused by cryptorchidism and the CFTR protein.[8]

Cryptorchidism is a congenital disorder in which the descent of the testis from the abdomen to the scrotal bag through the inguinal canal at birth does not occur. Patients with cryptorchidism experience infertility due to a failure in spermatogenesis. It is common knowledge that the temperature in the scrotal bag is approximately one or two degrees lower than that in the rest of the human body. Patients with cryptorchidism will have a higher temperature in the testicles.

In addition, CFTR is a temperature-sensitive protein. Low temperatures favor the correct assembly and maturation of the protein in the membrane, whereas high temperatures inhibit these processes and, as a result, reduce the total number of functional proteins in the membrane. For this reason, patients with cryptorchidism have fewer functional CFTR channels, which leads to an increase in the production of PGE2, alteration of the blood–testis barrier tight junctions, and, therefore, a failure in spermatogenesis.

CFTR and Its Role in Spermiogenesis

Spermiogenesis is the last phase of spermatogenesis that takes place from the spermatid stage until the release of mature spermatozoa. The haploid spermatids undergo a series of drastic morphological changes through a process of differentiation and specialization.

As it has been mentioned, the expression of CFTR in testes was determined by different research groups.[18,21] More specifically, there has been a differential expression of the CFTR protein in the spermatogonial cells at different stages of the spermatogenic cycle of rats. Most of this expression was detected in the round spermatids,[16,19] although in humans it was located in elongated spermatids and spermatocytes.[24]

In this way, the expression of CFTR in postmeiotic spermatocytes suggests the possible role of the protein in the process of spermiogenesis during which the spermatids differentiate into mature sperm.[16]

During spermiogenesis, a series of changes occur, such as condensation of chromatin and formation of the acrosome. In addition, a great cellular remodeling occurs in such a way that the round spermatids change their morphology, generating elongated spermatids, and finally, they generate the flagellate form through the loss of cytoplasmic mass. Therefore, the CFTR channel could be involved in the reduction of cell volume through the efflux of Cl^- and water and even through the interaction with other proteins of the membrane such as the AQPs.[4,8,16]

Currently, numerous AQPs have been identified in the testis, the efferent ducts, the epididymis, the vas deferens, and the accessory glands of adult mammals.[4,25] In addition, it was demonstrated that AQPs participate in early stages of spermatogenesis. The alteration of its expression and regulation is the basis of some types of male infertility.[25]

Moreover, there is scientific evidence of the possible role of the CFTR channel in spermatogenesis by the transport of HCO^- since the presence of the ACs in the spermatogonial stem cells was identified.[8] Therefore, as it happens in Sertoli cells, the influx of HCO^- activates the ACs in spermatogonial stem cells generating cAMP, which activates PKA. PKA, in the case of the spermatogonial stem cells, activates the "cAMP-responsive element modulator" (CREM) transcription factor.[8,20] It is known that the CREM transcription factor causes the activation of important genes in spermiogenesis.[20] As a result, an experiment was carried out in which a homozygous CFTR knockout mouse was compared with a wild-type mouse and a decrease in the levels of activation of CREM was observed in the knockout mice. Finally, it was concluded that CFTR in the cAMP/CREM via in spermatogonial stem cells and its role in the regulation of spermiogenesis are very important.[21]

CFTR and Its Role in Sperm Capacitation

Sperm capacitation is the process whereby sperm acquires the ability of fertilization. This process involves a series of modifications in spermatozoa: an increase in the intracellular Cl^- concentration, hyperpolarization in the membrane potential, an increase in pH and intracellular Ca^{2+}, and hyperactivated motility. In addition, sperm capacitation is a prerequisite to the acrosomal reaction, through which spermatozoa are able to penetrate and merge with the oocyte.[26,27]

Sperm capacitation occurs naturally along the female reproductive tract, which suggests the presence of certain molecules responsible for this phenomenon. Various experiments were carried out in such a way that the presence of HCO^- in high concentrations was identified in the female reproductive tract. Similarly, successive trials demonstrated the importance of HCO^- in the process of sperm capacitation and acrosomal reaction.[8,23]

As it has been mentioned, HCO^- causes activation of the sAC in sperm cytoplasm, leading to a series of events such as the production of cAMP and the phosphorylation of proteins through PKA, and consequently, the capacitation takes place.[27,28]

However, it has been demonstrated recently that Cl^- is also necessary for the processes associated with sperm capacitation mentioned earlier. In this way, the CFTR channel was proposed as a candidate to carry out simultaneous transportation of HCO^- and Cl^- inside the spermatozoa in the capacitation process.[26] In addition, clinical studies have shown that when the CFTR channel is inhibited, a blockage of the capacitation process occurs, and therefore, the involvement of the CFTR channel in that process is evident.[15,27,28] Furthermore, the CFTR channel acts in the regulation of other transporters such as the anion exchanger of Cl^- and HCO^- (SLC26A3). In addition, the colocalization of both transporters in the head and in the middle piece of the flagellum has been demonstrated, which gives scientific evidence of the possible interaction of the CFTR channel and transporters of the SLC26 family and their role in sperm capacitation.[23,26]

Similarly, subsequent studies have shown that anion exchange channels, such as SLC26A3 or TAT1, interact and cause the activation of the CFTR channel.[29] Therefore, a possible indirect mechanism was settled. In this mechanism, CFTR acts as a way of recycling Cl^- that generates the electrochemical gradient necessary to maintain the influx of HCO^- by the SLC26A3 (Figure 7.3). A research group demonstrated this mechanism because they noticed that without the correct CFTR function, the entrance of HCO^- was interrupted due to the absence of CI^- exchange, causing a failure in sperm capacitation.[23]

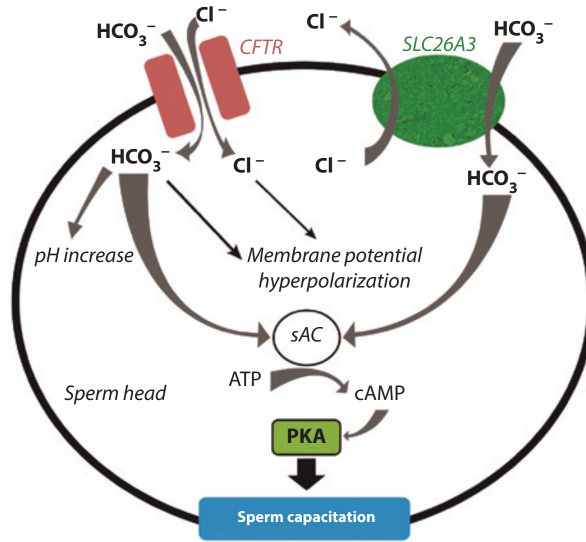

FIGURE 7.3 Activation pathway of cystic fibrosis transmembrane conductance regulator (CFTR)-dependent soluble adenylate cyclase (sAC) and SLC23A3 during sperm capacitation.

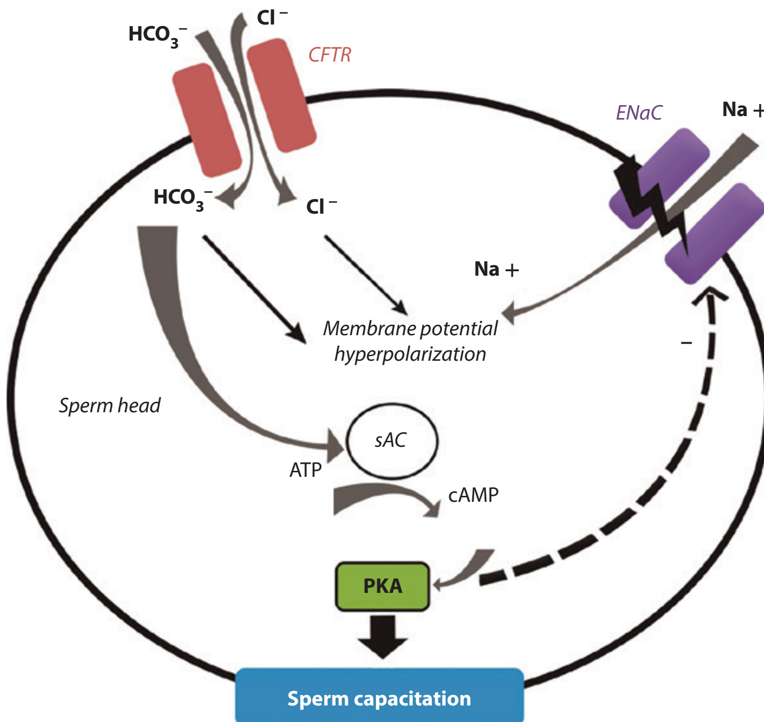

FIGURE 7.4 Inactivation or closing pathway of epithelial sodium channel (ENaC) through cystic fibrosis transmembrane conductance regulator (CFTR) activation, generating a membrane potential hyperpolarization during sperm capacitation.

At the same time, recent studies have suggested the participation of the CFTR channel in sperm capacitation through its interaction with the ENaC.[15,27] It was observed that the hyperpolarization of the membrane, which takes place during the sperm capacitation process, is generated by the closure of the ENaCs (Figure 7.4). In the same way, it was demonstrated that the activation of the CFTR channel

caused the inhibition of the ENaC, and therefore, it gave rise to the observed hyperpolarization during sperm capacitation.[27]

Finally, it has been proved that the CFTR channel participates in the secretion of Cl^- in response to neurohormonal factors in the distal region of the epididymis to promote the secretion of fluids for sperm transport, specifically during ejaculation.[8,30] Thus, mutations in the *CFTR* gene could be responsible for the absence of fluid secretion along the male reproductive tract and, consequently, cause an obstructive azoospermia where sperm cannot be transported outside.

As we have seen earlier, HCO^- plays an important role in sperm capacitation and also promotes the motility of spermatozoa in the epididymis region and the vas deferens before ejaculation.[31] Therefore, the CFTR expression along the epididymis seems to be important in the final maturation of sperm and its fertilization ability since a low expression of CFTR in the epididymis of infertile patients has been observed.[32]

Conclusion

Over the last decades, numerous research studies have been accumulating evidence of how involved the *CFTR* gene is in male infertility as it can be observed in diseases such as CF and BAVD. Different types of mutations of the same gene have been identified, giving rise to severe heterogeneous phenotypes. However, mutations of the *CFTR* gene are also found in patients with nonobstructive azoospermia, oligospermia, and teratospermia, which could clarify the potential role of the CFTR in the regulation of spermatogenesis and sperm capacitation, both important processes in male fertility.

Accordingly, these studies have clearly shown that CFTR not only acts as an ion channel that transports Cl^- and HCO^- but also behaves as a regulator of the function of other channels or transporters, which are involved in important processes to carry out spermatogenesis and sperm capacitation correctly.

The importance of CFTR in spermatogenesis, through its function in Sertoli cells, has been demonstrated. CFTR generates the activation of genes involved in that process through the sAC/cAMP/PKA/CREB signaling pathway. In addition, CFTR also acts by inhibiting the NF-κβ/COX-2/PGE2 (cyclooxygenase-2) signaling pathway. This pathway helps maintain the stability of the blood–testis barrier that is important for the development of a proper spermatogenesis.

The findings mentioned throughout this chapter provide evidence of the possible molecular mechanisms by which mutations in the *CFTR* gene would have different types of observed male infertility as a consequence, as it happens in patients with CF, BAVD, teratospermia, nonobstructive azoospermia, and oligospermia. In this way, it is interesting to consider the potential use of the *CFTR* gene as a possible biomarker of male infertility, not only as a genetic risk for conceiving children affected by cystic fibrosis. In fact, there is a high correlation between mutations of the *CFTR* gene and semen quality,[15] that is, there is a higher percentage of protein expression in fertile men whereas in infertile men with teratospermia or asthenospermia, the expression of CFTR is hardly observed. These results suggest that the percentage of CFTR protein expression could be used as an indicator of the quality of semen and its fertilization ability.

In addition, there are scientific studies that indicate that it is important to test mutations of the *CFTR* gene in every patient with nonobstructive azoospermia, oligospermia, or with low seminal quality regardless of whether they have CF or BAVD.[33] At the same time, the detection of these mutations would be very useful and important in assisted reproduction treatments to avoid that offspring inherit CFTR mutant alleles and can develop some type of infertility, or at the same time, they can pass it on to their own offspring.

In addition, as it is known, CFTR regulates the function of other transporters involved in reproductive processes. Thus, these transporters, such as SLC26A3, different AQPs, and the ENaC, could be used as biomarkers of male infertility to complete the diagnosis. Accordingly, it would be interesting to carry out an exhaustive study of all possible candidate genes whose mutations give rise to spermatogenesis failures and lead to poor sperm quality and, as a result, an inability of the sperm to fertilize the oocyte. Subsequently, the implementation of a genetic test to compare the genomic DNA with a panel of mutations in different genes involved in the earlier-mentioned processes could be very helpful in the diagnosis of patients before a cycle of assisted reproduction.

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The Application of Genetic Tests in an Assisted Reproduction Unit: mRNA Microarrays

Sandra Garcia

Introduction

In developed countries, infertility problems affect around 7%–8% of all couples, this percentage increased to 15% of couples needing assisted reproduction[1] (of which approximately 50% have altered male fertility profiles)[2] in Europe. The other half of these couples do not present problems when submitted to ordinary evaluation processes and infertility work-up, but are still unable to achieve pregnancy, even when the female partner is young and healthy. These are referred to as cases of idiopathic male infertility, a subset of patients in whom the basic semen analysis is unable to detect motile sperm production alterations, which some reports estimate count for up to 40% of subfertile men.[3] The fact when sperm analysis is normal in infertile males but they are diagnosed as presenting idiopathic infertility[4] clearly points to the need for more accurate fertility markers to predict a successful pregnancy, either by means of natural conception or assisted reproduction. However, this is a complex issue, given that the markers studied in one of the partners aim to predict the results obtained by the interaction of the male and female gametes.

Microscopic evaluation of male reproductive ability is the first male requirement to undergo assisted reproduction treatments, and the only accepted tool to estimate the fertility male potential is the basic sperm analysis as stated by the World Health Organization (2010).[5] Male fertility is considered optimal when an adequate number of normal shape, motile, mature, and physiologically functional sperm are produced. This means that sperm quality evaluation should consider both microscopically visible features and the molecular capacity to perform several physiological processes such as swimming through the female reproductive tract, crossing the cervical mucus, capacitation, zona pellucida recognition, and binding, acrosome reaction, entering, and activating the oocyte, and, ultimately, conferring its DNA, messenger RNA (mRNA), and structures[6–8] (Figure 8.1) to the oocyte to create a normal embryo that is capable of developing, implanting, and growing until delivery as a healthy baby. The molecules provided by fertilizing sperm are crucial until the stage at which the embryo's genetic machinery, obtained from the mother's and father's genomes, takes control of its fate[9]; nevertheless, the classic sperm analysis stated by the World Health Organization (WHO) has limited predictive power to forecast pregnancy because it is not able to evaluate molecular features involved in those several physiological processes useful for assessing sperm reproductive ability and needed to reach a successful pregnancy.[10,11] The efficacy of semen analyses in assessing male fertility is the subject of a hot debate that revolves around a central concept: the necessity to develop new markers of sperm function.[11,13] Furthermore, all these assertions also may be considered from the point of view of a single ejaculate. In this sense, it is interesting to define a sperm sample as able to accomplish a pregnancy or not, instead of defining a male as fertile or infertile.[14]

Until now, assisted reproduction units have tended to evaluate sperm quality only from the cellular view point, with very little information having been gathered about molecular sperm components. Many, but not all, causes of fertility correlate closely with cellular features of the ejaculate. Except from chromosomal aneuploidies,[15–17] or Y-chromosome microdeletion investigations,[18] which are both linked

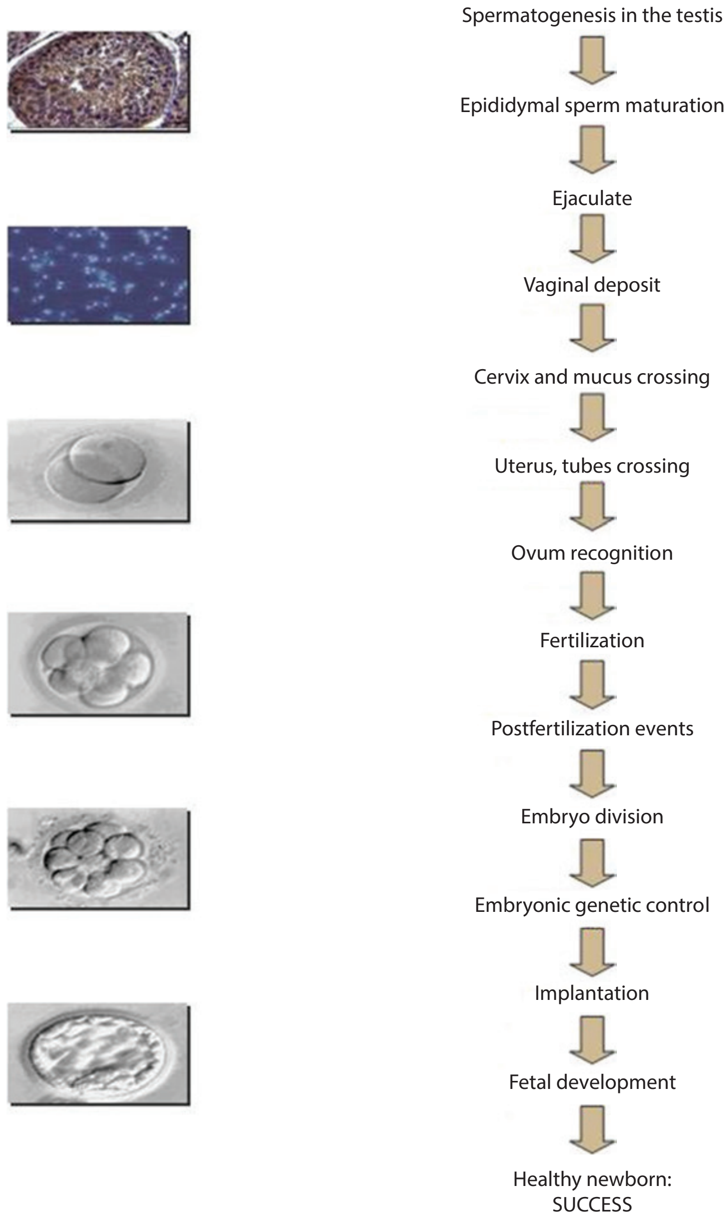

FIGURE 8.1 Flow chart of reproduction processes that spermatozoa need to overcome to reach a successful pregnancy. (Courtesy of Dr. N. Garrido.)

to severe alterations in sperm count, no molecular features of sperm complementing the basic sperm analysis are available as a diagnostic tool.

Among molecular infertility markers, contradictory data have been found in the literature regarding the usefulness of sperm DNA integrity analysis.[19,20] Oxidative stress (OS) in spermatozoa has also been exhaustively studied during the last decades as a potential male fertility marker aiming to predict male fertility.[21,22]

Other molecular markers, not related to those above, have been tested in spermatozoa to predict their ability to accomplish fertilization and pregnancy,[14,23,24] and all evidence demonstrates the multifactorial origin of sperm dysfunction[8] (Figure 8.2).

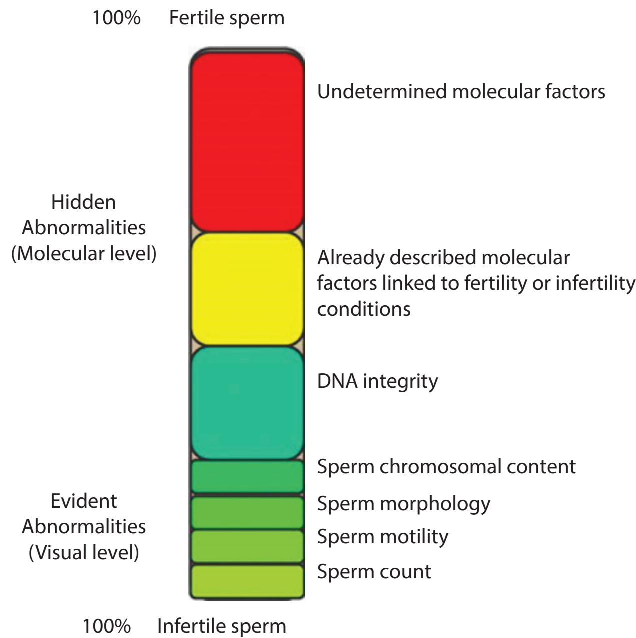

FIGURE 8.2 Diagram of all the factors that could be involved in male fertility potential. (Courtesy of Dr. N. Garrido.)

Analytical Techniques: Microarray Technology as a Diagnostic Tool

Microarrays and other innovative technologies are a wide range of new techniques that are used to unveil causes of infertility, for example, the Polscope and atomic force microscopy for the detailed study of sperm morphology and structure, and microfluidics that quickly isolates good morphology spermatozoa from poor-quality spermatozoa.[24] Today, it is well known that, as well as supplying DNA, the spermatozoon also provides the egg with paternal centrioles, proteins, and RNA.[25] Although the functional significance of mRNA in mature spermatozoa remains essentially unexplored,[26] it is known that sperm mRNA is necessary from the moment of the first embryo cleavage until the activation of the embryonic genome[27] and may influence the phenotypic traits of the embryo[28] and offspring.[29] Since its introduction in 1994, microarray technology has made significant advances in the identification and characterization of novel or known genes correlated with male infertility in mice and humans.[30]

Molecular biology techniques as microarrays allow the analysis of thousands of genes, proteins, or metabolites at a time and have allowed the scale of biologic research to be expanded from the study of single genes or proteins to a systematic study of all genes and proteins. They provide a global view of biological processes, being of particular interest to molecular medicine, where they have thousands of potential applications. Among the different available ''omics'' platforms, complementary DNA (cDNA) microarrays, which measure mRNA transcript levels, are of particular interest given the previously mentioned relevance of paternal mRNA for reproductive success. Transcriptomics is the study of the mRNA pool expressed in a cell at a particular time under determined conditions.[31] This technique has recently been applied to sperm evaluation.[8,32–34]

These platforms provide a list of genes whose expression has been detected in a sample, pool, or group, as well as the fold change or exclusivity of gene expression when two biological situations are compared. With the help of different bioinformatic tools, an ontological interpretation of the results can be obtained from current molecular knowledge and databases, which include lists of cellular components, biological processes, and molecular functions that are regulated by a significant number of the genes that are underexpressed or overexpressed.[32–34]

The aim of this chapter is to deepen our knowledge about one of those sperm molecular feature candidates to be involved in pregnancy success: the mRNA content (transcriptome) of spermatozoa that

achieved successful fertilization and implantation versus spermatozoa that did not achieve successful fertilization and implantation in different assistive reproductive technologies (ARTs).

The Scientific and Biological Bases of Microarray Test: Involvement in Reproductive Function

mRNA Content in Spermatozoa: Evidences

The existence of a complex population of mRNAs in human sperm is well documented. It is currently accepted that sperm cells are not mere paternal genome carriers; as well as providing DNA, sperm cells also supply the egg with the paternal centrosome, which forms an aster of radially arrayed microtubules that ease the fusion of male and female pronuclei, organize the first mitotic spindle in the zygote,[35] and activate the soluble oocyte-activating factors that induce calcium response (e.g., phospholipase C protein)[36–38] and various transcription factors and signaling molecules essential for fertilization and embryo growth (e.g., signal transducer and activator of transcription 4). Sperm also contain a set of stable and functionally active mRNAs that are relevant for reproductive success.[25–27,29,39]

The male gamete is transcriptionally silent as a consequence of the highly condensed architecture of its chromatin and because there is little or no cytoplasm capable of withstanding translation. It was originally hypothesized that the RNA present in sperm was related to contamination from somatic cells. However, RNA is present after stringent washing through density gradients, which shows that it originates in the sperm fraction and is subsequently introduced into the oocyte during fertilization.[40] Furthermore, despite the presence of a cell type that suffers a transcriptional shutdown, several reports by different authors have described the presence of mRNAs in sperm cells measured by means of mRNA in situ hybridization, RNA display, or reverse transcription polymerase chain reaction (RT-PCR) techniques. The presence of RNA in ejaculated spermatozoa has been described in several species, and a number of different molecules have been located within the nucleus, midpiece, or tail,[37] including c-MYC,[41] heat shock proteins 70, 90, and beta-actin,[42] human leukocyte antigens,[43] L-type calcium channels, N-cadherin,[44] estrogen receptors,[45] cyclic-nucleotide phosphodiesterases,[46] integrins, aromatase, and nitric oxide synthase (NOS),[47] glutathione peroxidases 1 and 4, and glutathione reductases,[28,48,49] transcription factors NF-êB (Nuclear fator kB), HOX2A (homeobox A2), ICSBP (interferon regulatory factor 8), protein kinase JNK2 c-Jun N-terminal protein kinase 2), growth factor HBEGF (heparin binding EGF like growth factor), and receptors RXRb (retinoid X receptor beta) and ErbB3 (erb-b2 receptor tyrosine kinase 3) within the nucleus,[50,51] among others.

Even if the functions of most of sperm mRNAs are still unknown, those of others are well documented in the literature. For example, stable mRNAs are known to survive the different stages of spermatogenesis prior to nuclear silencing, even DNA packaging, in an analogous situation to that described in oocytes.[9,29] These mRNAs have been related with successful syngamy and embryo development,[52] and certain mRNAs described in the spermatozoa have been found to be lacking in oocytes.[27] In view of such evidence, it may be considered that some mRNAs must be necessary for the development just before embryonic genome activation.

Other possible functions of these molecules include de novo translational replacement of degraded proteins and epigenetics regulation (establishment/maintenance of parental imprint modifications).[29] In addition, the transcriptional activity has been described in mitochondria; mRNAs are able to move out of the nucleus and bypass nuclear shutdown, thus remaining functional in other cell locations.[29]

The main advantage of microarrays experiments is also one of its main drawbacks; as a result of mRNA microarray analysis of the whole genome, tens of hundreds of mRNA are detected. This large amount of information is difficult to classify because changes in transcript levels result in long lists of genes. Nevertheless, these biological changes do not occur as independent events, as such lists suggest, but rather as a highly coordinated and interdependent process.[53,54] In order to provide a functional interpretation of those long mRNA lists, an ontology analysis could be performed showing a broad range of ontologies, which suggests that mRNA lists perform a high variety of functions. These functions, in genes expressed early on in spermatogenesis, and in fertilization and early embryonic development

(many of them absent in unfertilized oocytes), are thought to include signal transduction, oncogenesis, cell proliferation from plasma, and nuclear proteins.[39,55]

One of the hypothetical functions of mRNAs is the synthesis of proteins involved in the repackaging of DNA and other transcripts required at a later stage to surpass the temporal barrier where spermatocytes segregate transcription from translation and store messages are required for later events in spermiogenesis. This subset also includes micro-RNAs, whose function is still far from understood. These are small noncoding RNAs with between 19 and 23 nucleotides and which are known regulators of posttranscriptional translation at every stage of spermatogenesis, including, for instance, spermatid differentiation.[56]

The evidence available suggests that, whether spermatozoal RNA is residual or has a key role in reproductive function, it offers the potential to improve the investigative and diagnostic potential of semen profiling and could provide molecular signatures of the male infertile phenotype. In this way, it can be used to explain cases of idiopathic infertility with normal spermiogram, in which reproduction is not achieved, thus representing a potential diagnostic tool.[13]

Sperm Molecular Feature Required in ARTs

The scientific literature tells us that in sperm cells there is a complex mRNA population (also known as transcriptome) that could determine fertile male potential, so the mRNA profile or signature must differ between fertile and infertile males.

To evaluate if there were differences in mRNA content between infertile and fertile men, our group compared mRNA population in infertile patients undergoing ARTs and proven fertile sperm donors.[33,57]

Once those differences were reported, the next step was to compare if the molecular sperm features required were different depending on the ARTs used.

The current ARTs used by patients with fertility problems could be divided into three categories in terms from less to more complexity and level of human intervention: intrauterine homologous insemination (IUI) (procedure in which a fine catheter [tube] is inserted through the cervix into the uterus to deposit a sperm sample from the woman's mate directly into the uterus), *in vitro* fertilization (IVF) (procedure in which oocytes from a woman's ovary are removed and fertilization took place in a dish where many sperm are placed near an oocyte, and then the embryo is returned to the woman's uterus), and intracytoplasmic sperm injection (ICSI) (procedure in which oocytes from a woman's ovary are removed and the embryologist selects a single sperm to be injected directly into an oocyte to fertilized it, then the embryo is returned to the woman's uterus). It has been suggested that the molecular requirements for sperm to achieve a pregnancy are not the same for all ARTs. There are less functional requirements for sperm to succeed when the reproductive techniques are more invasive and allow several natural processes to be bypassed. This implies that the ''molecular machinery'' used by sperm cells is necessary or unnecessary, depending on the ART used.[8] For example, in the case of IUI versus ICSI, successful sperm will require more molecular capacity to fulfill their mission in the former case, as all the previous steps of the fertilization process—even oocyte/sperm interaction—are skipped with ICSI. In accordance with this, different gene expression profiles are expected.

mRNA Profile Donors versus Patients

As mentioned, our group first compared mRNA population in infertile patients undergoing ARTs and proven fertile sperm donors.[57]

After obtaining written consent from each patient, 10 sperm samples were obtained from strictly selected infertile men (five samples, one per man) from couples attending our infertility clinic after 1 year of unprotected regular intercourses (mean infertility length was 1.5 years), presenting normal sperm count and motility (WHO criteria) parameters, where no infertility cause was observed in their partners' (ages <35 years) routine work-ups, and sperm donors (five samples, one per donor) of proven fertility, demonstrated by their declaration of having their own children and having had healthy newborns in our sperm donation program.

These men with fever episodes, genital tract inflammation, varicocele, recurrent infections, exposure to toxins, or other similar situations leading to confounding results were not accepted in the study. These data were obtained by a directed questionnaire.

Sperm samples were obtained in our facilities and directly transported to the Andrology Laboratory. After liquefaction, a basic sperm analysis was done as described elsewhere.[28] No differences were found

in any of the sperm parameters between groups, i.e., sperm concentration, motility, and morphology. Mean age was similar between groups.

The total number of sperm collected for the experiments was standardized to 50 million to provide enough spermatozoa for all tests, concentrating by centrifugation for 10 minutes at 400 × *g* and eliminating the supernatant. Then sperm samples were immediately frozen by direct immersion in liquid nitrogen until the total numbers of samples programmed for this study were obtained and experiments were performed (less than 1 week).

Sperm mRNA was extracted using Trizol protocol (TelTest, Friendswood, TX), suspended in diethylpyrocarbonate (DEPC)-treated water and frozen at −80°C until the microarray experiments were performed in duplicate. The total amount of RNA was quantified by spectrophotometry on a BioRad (Durviz, Valencia, Spain) spectrophotometer.

Equal amounts of RNA from the same groups were pooled before the analysis, as recommended for short series.[55] CodeLink Expression Analysis System was used according to the manufacturer's instructions. Human Whole Genome Bioarray contains probes for more than 55,000 gene targets. Comparisons between the two groups were performed in duplicate. Spot intensities were normalized and analyzed using the CodeLink Expression Analysis v4.1 software.[58]

mRNA Profile in IUI

A significant percentage of couples (30%–40%) fail to achieve pregnancy despite several IUI attempts, even in cases without a clear male infertility factor, which suggests the existence of an occult cause of male infertility that has lately been linked to molecular factors, but not to sperm count, motility, or morphology.[59]

Sperm samples were obtained from selected couples undergoing IUI (only one cycle per couple). Twenty sperm samples were included in the study, 10 from men whose sperm initiated a pregnancy and 10 from men whose sperm failed to do so (Figure 8.3). The objective was to establish if mRNA content could determine a successful IUI treatment; for that reason all sperm samples presented similar sperm parameters as stated by WHO, with sperm motility higher than 25%, sperm density of 10 million/mL, and more than 3 million

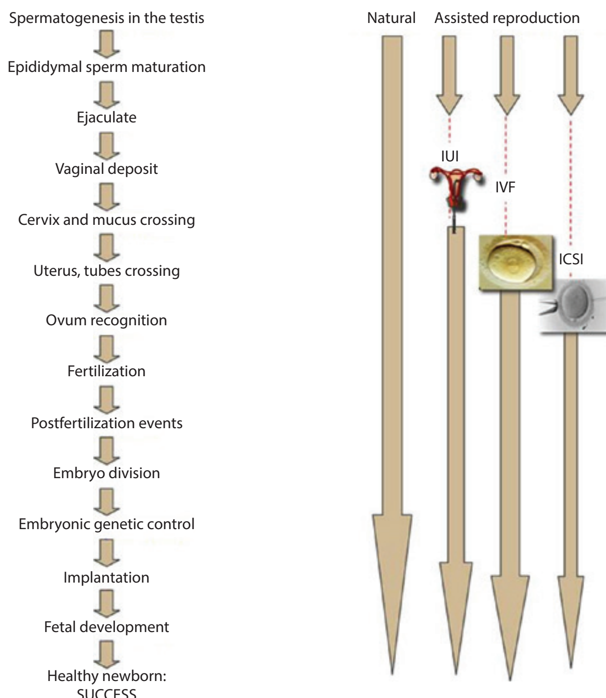

FIGURE 8.3 Flow chart of natural reproduction processes by-passed by spermatozoa depending on assisted reproductive technologies (ARTs) used. (Courtesy of Dr. N. Garrido.)

motile sperm after preparation. In fact all the sperm samples were catalogued as "normal" following the WHO criteria. Furthermore, female partners must be under 36 years old and not present endometriosis and polycystic ovarian syndrome; their tubal permeability was determined by hysterosalpingographic examination. All men maintained 3–5 days of sexual abstinence before the sperm sample was obtained.

Approximately 100–200 aliquots microliters were retrieved from sperm samples that subsequently were processed and used in the IUI procedure. Briefly, aliquots of the sperm samples were washed in 3 mL of phosphate-buffered saline and centrifuged for 10 minutes at 300 *g*. After discarding the supernatant, the pellet was resuspended in 1 mL of TRizol (Invitrogen, Barcelona, Spain) and immediately frozen by direct immersion in liquid nitrogen. The pellet was then stored in a nitrogen tank until mRNA extraction. Total RNA was extracted using the TRIzol method according to the protocol recommended by the manufacturer (Life Technologies, Inc., Gaithersburg, MD)[28,60] (Figure 8.4).

Once we identified patients who got pregnant and those who did not, samples from 10 infertile patients who achieved pregnancy (group P) and 10 who did not (group NP) by IUI treatment were pooled with equal amounts of RNA, and microarray experiment was performed. Finally, four microarrays were performed: two in duplicate for both groups P and NP (Figure 8.5).[10]

We considered the microarray results from different approaches to evaluate our hypothesis, which holds that sperm mRNAs from samples achieving pregnancies after IUI treatments with healthy and apparently fertile females exhibit a different transcriptome from samples unable to achieve pregnancy (Figure 8.6).

Differentially Expressed Transcripts

Three criteria were used to define differentially expressed transcript (DET) in the different sample sets: transcripts that were common to groups P and NP, showed an absolute fold change expression (FC) of twofold or more and a corresponding p-value less than 0.05 ($p < 0.05$). Positive FC values reflect an overexpression in P and NP, and a negative value denotes overexpression in NP and P.

Exclusive Expressed Transcripts

Another method of analyzing microarray data is to detect those transcripts or sequences that are expressed in only one of the groups and absent from the other: the exclusive transcripts (EETs). Two criteria were used to define EET: spot intensity level greater than the mean of the density of the negative control plus 2 ±SD and to be exclusively expressed only in one of the two groups. In our experiments, this mean intensity was 6.78 units.

Gene Ontology Analysis

Gene ontology (GO) analysis has been used to provide structured knowledge.[33,61] The GO was designed as a formal representation of biological knowledge as it relates to genes and gene products.[62] It consists of three knowledge domains (GO terms): molecular function, biological process, and cellular component.[63] The data generated by the software analysis of the scanned array images (DET and EET lists) were imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp) to detect activations or inactivations of biological processes, molecular functions, or cellular components.[64] In short, GO analysis gives biological meaning to the mRNA list provided by microarray.[65,66]

Genes Previously Described in Reproduction-Related Biological Processes

Data on transcripts involved in biological processes that might be related to male fertility were accessed by searching systematically for key GO terms as follows: acrosome (GO term 0001669), acrosome reaction (0007340), sperm binding to zona pellucida (0007339), copulation (0007620), embryo implantation (0007566), embryonic development (0009790), female pregnancy (0007565), fertilization (0009566), male gamete generation (0048416), genitalia development (0048806), germ cell development (0007281), gonad development (0008406), insemination (0007320), mating (0007618), placenta development (0001890), reproduction (0000003), reproductive process (0022414), sexual reproduction (0019953), acrosomal vesicle (0001669), sperm motility (0030317), spermatid development (0007286), spermatid nucleus differentiation (0007289), spermatogenesis exchange of chromosomal proteins (0035093), and spermatogenesis (0007283).

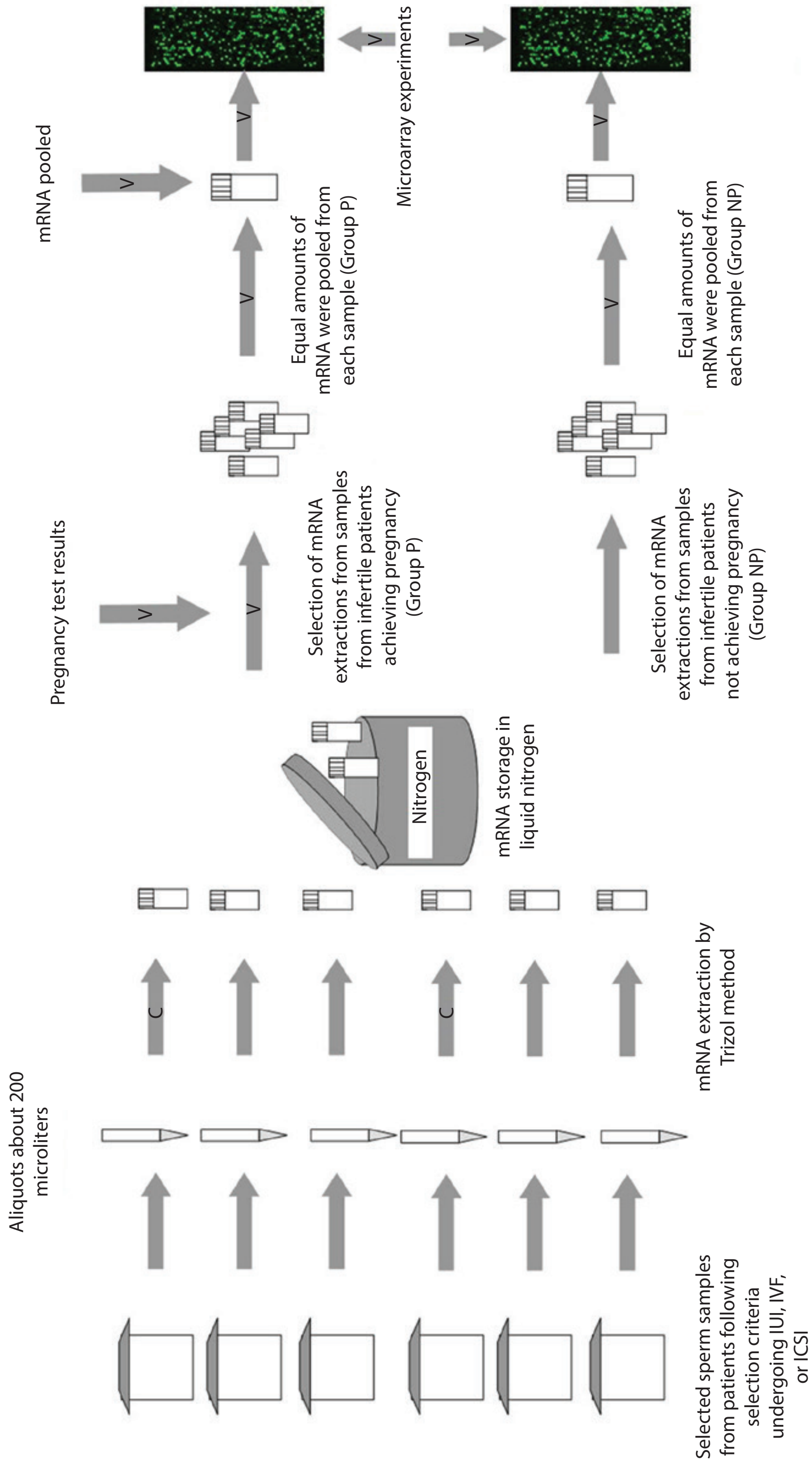

FIGURE 8.4 Samples processing and storage, general flow chart.

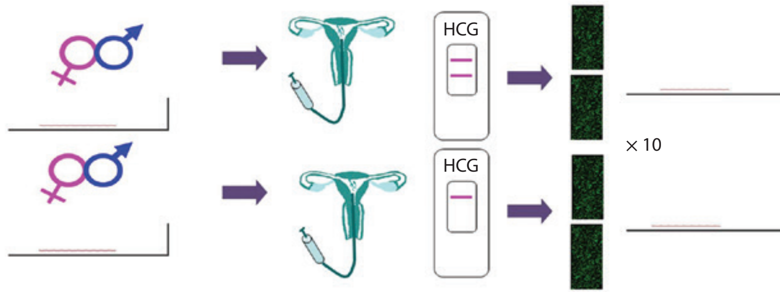

FIGURE 8.5 Intrauterine homologous insemination (IUI) microarray experiments, flow chart. Couple A gets pregnant and couple B does not. A total of 10 samples per group (pregnancy [P] and nonpregnancy [NP] groups) were selected and pooled to perform microarrays.

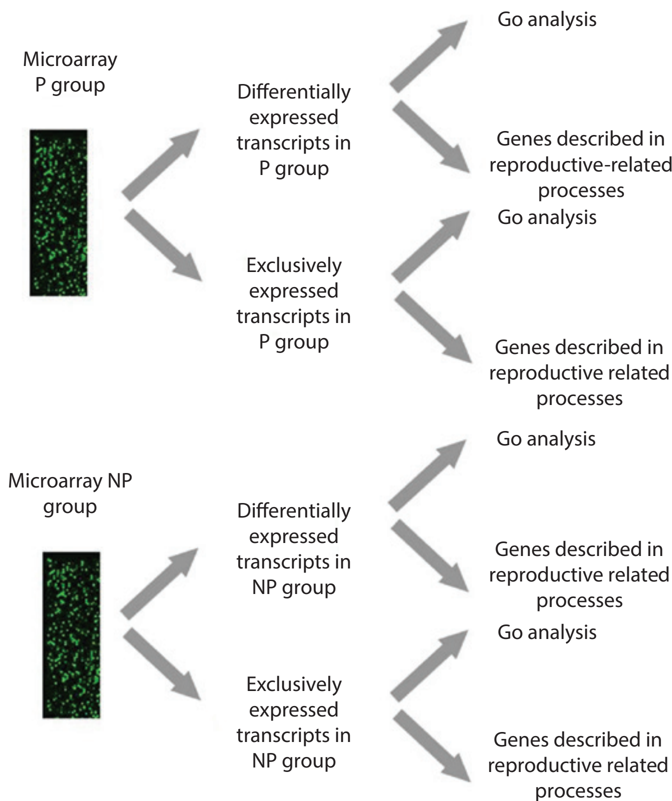

FIGURE 8.6 Microarray data (differentially expressed transcripts [DET] and exclusive expressed transcripts [EET]) analysis, flow chart.

A database of transcripts associated with reproduction-related biological processes was created from those detected by the GO analysis and compared with the study's lists of DET and EET for sperm samples from groups P and NP.

mRNA Profile in IVF

To establish mRNA profiles in spermatozoa from patients undergoing IVF we analyzed whether the spermatozoa couples who received oocytes from proven fertile donor became pregnant after the IVF procedure. Eight couples were chosen in which fresh spermatozoa were used. Four of them achieved pregnancy (group P, $n = 4$) and four did not (group NP, $n = 4$).

The inclusion criteria for women were the following: 30–45 years of age, first IVF cycle, and body mass index <30 kg/m^2. Transfers were performed approximately 72 hours after oocyte retrieval. Exclusion criteria were the presence of a uterine pathology (adenomyosis or uterine dysfunctions), significant endocrinological or metabolic dysfunctions, and previous recurrent pregnancy loss.

To avoid female infertility as a bias factor in this study, we established a model that included design features new to the literature: the sperm mRNA transcriptome was determined by analyzing aliquots of an ejaculated sample used for assisted reproduction treatment; the variability of oocyte quality was reduced by restricting the study to a fresh ovum donation program, using fertility-proven oocyte donors who had been mothers on their own or previously as part of the donor program.

Microarray experiment samples from four infertile patients who achieved pregnancy (group P) and four that did not (group NP) by IVF treatment were pooled with equal amounts of RNAs, and microarray experiment was performed (Figure 8.7).

Microarray results were assessed with different approaches as described below in IUI cases, defining both DET and ET lists as well as the GO analysis.

mRNA Profile in ICSI

To establish mRNA profile in spermatozoa from patients undergoing ICSI we analyzed the spermatozoa of sets of two couples who received oocytes from the same donor but in which only one couple became pregnant after ICSI. Five sets of two couples were chosen in which fresh spermatozoa (n = 10) were used (Figure 8.8).

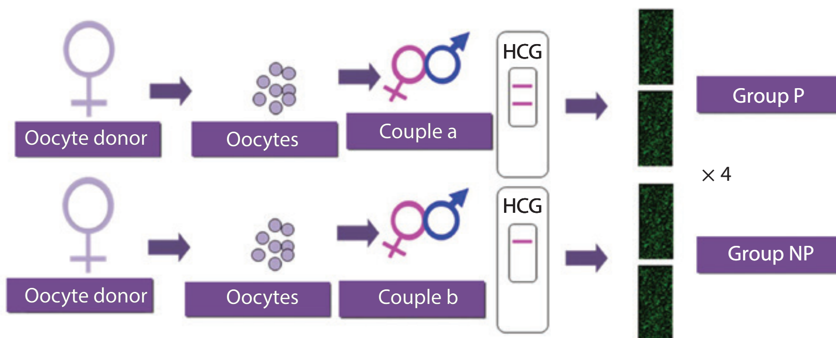

FIGURE 8.7 In vitro fertilization (IVF) microarray experiments, flow chart. Couple A gets pregnant and couple B does not with donated oocytes. A total of four samples per group (P and NP groups) were selected and pooled to perform microarrays.

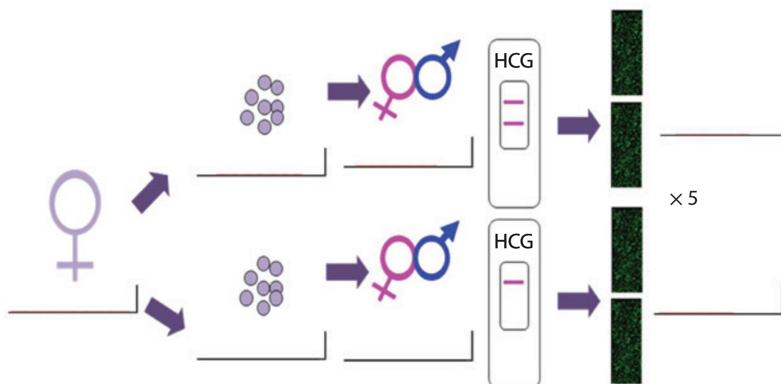

FIGURE 8.8 Intracytoplasmic sperm injection (ICSI) microarray experiments, flow chart. For this experiment we chose cases in which couple A gets pregnant and couple B does not using the same donor and oocyte cohort. Finally, five cases were selected so a total of five samples per group (pregnancy [P] and nonpregnancy [NP] group) were selected and pooled to perform microarrays.

The inclusion criteria for women were the following: 30–45 years of age, first ICSI cycle, and body mass index <30 kg/m^2. Transfers were performed approximately 72 hours after oocyte retrieval. Exclusion criteria were the presence of a uterine pathology (adenomyosis or uterine dysfunctions), significant endocrinological or metabolic dysfunctions, and previous recurrent pregnancy loss.

In an attempt to go beyond to avoid female infertility as a bias factor, another design feature was included and added to those described below in IVF procedure (the sperm mRNA transcriptome was determined by analyzing aliquots of an ejaculated sample used for assisted reproduction treatment as well as the use of oocyte from the donor program): sperm mRNA expression profiles were compared by grouping pairs of sperm samples associated with the same oocyte donor. This model allows different mRNA patterns to be partially associated with a successful pregnancy. Couples were assigned to either group P (pregnant) or group NP (not pregnant) according to the treatment outcome.

Microarray results were assessed using different approaches as is described below in IUI cases, defining both DET and ET lists as well as the GO analysis.[32]

Results

Both our data, as per previous literature, show that the spermatozoon is more than a paternal DNA carrier supplying a future embryo with centrioles, proteins, and mRNA pool[25] necessary from the moment of the first embryo cleavage until the activation of the embryonic genome[27] and may influence the phenotypic traits of the embryo[28] and offspring, although its functional role remains essentially unexplored.[26]

Based on the findings and according to previously published data about mRNA role in male fertility and reproduction, the working hypothesis is that sperm cells with and without reproductive success present different transcriptomes or RNA populations, resulting in different molecular requirements necessary to succeed depending on the assisted reproduction treatment used, seems to be well established.

Finally, our results showed tens to hundreds of genes that were differentially or exclusively expressed in the different study groups (P and NP groups), representing the three main ARTs: IUI, IVF, and ICSI (Figure 8.9).[10,32–34] In this way, we defined the signature or fingerprint of reproductive successful samples

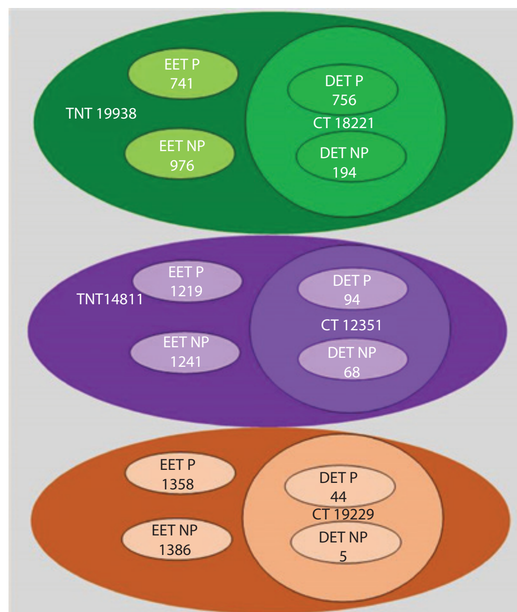

FIGURE 8.9 The number of different transcripts detected in each assisted reproductive technologies (ARTs) microarray experimenst. TNT, total number of transcripts detected in the microarray experiment; EET, exclusive expressed transcripts; DET, differentially expressed transcripts; CT, common transcripts to both groups.

for each treatment, thus creating the basis for developing a custom microarray-based diagnostic tool for sperm that are currently awaiting clinical validation.

These data also support the initial hypothesis that molecular requirements are less as more complex degree ARTs is (for example, IA requires less manipulation than an ICSI procedure), in order that DET involved in P groups decreased as the procedure became more invasive, as well as to became pregnant requires a more complex molecular machinery than not to get pregnant in order that in all studied ARTs DET in P groups are more numerous than in NP groups.

Apart from sperm provided by microarrays, further insight has been provided by these experiments. As shown in Figure 8.11, which reflects the most differentially expressed genes in each group and the genes expressed exclusively in a single group, the major differences in expression have been characterized in the entire genome. Many of these differences have been related for the first time to fertility, whereas other data obtained have confirmed previous findings.[32–34] The gene expression profile of sperm cells with reproductive success/failure varies with each ART technique in order than only a low percentage of DET needed to get o not pregnant from de different ARTs are common, nevertheless, more similar the ARTs are respect invasive degree more common DET they share (IUI is more similar to IVF than ICSI, as well IVF is more similar to ICSI than to IUI) (Figure 8.10).

Differential expression of a gene in one group with respect to another represents varying transcription levels but does not provide information about which way (up or down) a gene is regulated or how it can be translated to a biological role. We can only make assumptions based on previous research about those genes. Microarray analysis essentially provides us with a very long list of genes that are known to have significantly different transcript levels. However, in biology, these variations do not occur as independent events and it is improbable that a single gene can explain biological differences. For this reason, an ontological analysis of transcriptional differences is required.

We have obtained information regarding GO revealed by lists of differentially and exclusively expressed genes for each procedure, which depict a number of cellular components, biological processes, and molecular functions. This analysis revealed significant differences at both transcriptional and functional levels between spermatozoa that achieved pregnancy and those that did not. GO analysis can provide information about processes in which a significant number of genes are altered, suggesting a defective pathway in the physiology of certain sperm samples. Furthermore, the ontology analysis allowed the search for more fertility markers.

From GO analysis of different lists obtained in each ART the conclusion is as follows: the majority of GO terms (cellular components, biological processes, and molecular functions) that are statistically affected could be denominated as “general” GO terms as none or only a few of them could be directly related to fertility (i.e., spermatogenesis, reproduction, male gamete generation). The majority are

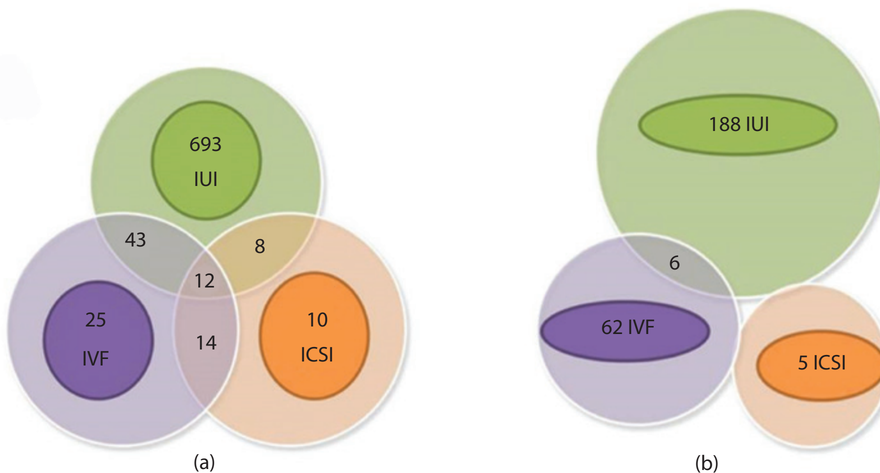

FIGURE 8.10 Common number of differentially expressed transcripts (DET) among the three techniques in both pregnancy (P) (a) and nonpregnancy (NP) groups (b).

Differentially Expressed Transcripts							Exclusive Transcripts		
Name	GenBank#	Fold Change	*p*-Value				Name	GenBank#	Fluorescence value
FCGR3A	NM_000569	20.71	0	Overexpressed in P group	Intrauterine insemination	Exclusive expressed in P group	IL8RA	NM_000634	9.916
OSM	NM_020530	19.6	0				CYSLTR2	NM_020377	9.732
FPR1	NM_002029	18.49	0				AK055428	AK055428	9.647
CXCL1	NM_001511	17.83	0				P2RY14	NM_014879	9.517
AQP9	NM_020980	17.75	0				C8orf39	AF116672	9.5
TMEM154	NM_152680	16.21	0.0005				PRG1	NM_002727	9.372
PI3	NM_002638	15.96	0.0007				IFIT2	NM_001547	9.344
LILRB2	NM_005874	15.35	0				BTNL8	NM_001040462	9.218
VNN2	NM_004665	15.22	0				STK4	NM_006282	9.175
APOBEC3A	NM_145699	14.1	0.004				SIKE	NM_025073	9.054
AK000872	AK000872	5.42	0.0254	Overexpressed in NP group		Exclusive expressed in NP group	THC2638360	THC2638360	10.428
NST000003036E	NST0000030369	5.13	0.0266				CDC37L1	NM_017913	10.382
ZNF224	BC002889	4.68	0				THC2547195	THC2547195	9.875
FLJ20581	NST0000033184	4.55	0.0133				NOX1	NM_007052	9.461
CAMK2N2	NM_033259	4.45	0.0097				CLCNKB	NM_000085	9.147
CGB1	NM_033377	4.26	0.0166				THC2555234	THC2555234	9.069
THC2541992	THC2541992	3.98	0.0135				THC2648250	THC2648250	9.022
THC2609820	THC2609820	3.87	0.015				NETO1	NM_138966	8.873
LCE2D	NM_178430	3.75	0.0082				THRSP	NM_003251	8.815
VPS18	NM_020857	3.7	0.0101				AADAC	NM_001086	8.264

FIGURE 8.11 DET and EET Lists from P and NP Groups from Different ARTs. *(Continued)*

Differentially Expressed Transcripts							Exclusive Transcripts		
Name	GenBank#	Fold Change	*p*-Value				Name	GenBank#	Fluorescence value
C10orf119	NM_024834	34.27	0.006	Overexpressed in P group	In vitro fertilization	Exclusive expressed in P group	MMP12	NM_002426	9.276
SPP1	NM_001040058	32.72	0.01				C10orf119	NM_024834	9.252
TGFBI	NM_000358	17.44	0.005				CLEC4E	NM_014358	8.602
CD163	NM_004244	15.24	0.0033				ADAMDEC1	NM_014479	8.526
ADM	NM_001124	14.34	0.0025				PTPN12	NM_002835	8.248
RGS2	NM_002923	13.98	0.002				PLEK	NM_002664	8.018
MMP9	NM_004994	13.66	0.005				CXCR7	NM_001047841	7.87
CTSL	NM_001912	13.56	0.0043				MAP3K8	NM_005204	7.805
MT1M	NM_176870	12.91	0.0038				LYZ	NM_000239	7.788
IFI30	NM_006332	12.82	0.003				INDO	NM_002164	7.734
PLA2G2A	NM_000300	42.31	0.0194	Overexpressed in NP group		Exclusive expressed in NP group	C10orf64	BC034937	8.369
SH3RF2	NM_152550	8.05	0				MSH4	NM_002440	7.969
LELP1	NM_001010857	7.67	0.005				DCC	NM_005215	7.106
HSPA1L	NM_005527	5.76	0.0125				MYH7	NM_000257	7.031
DUSP21	NM_022076	5.5	0.01				INPP5F	NM_014937	7.008
TSPAN16	NM_012466	5.42	0.01				C4orf6	NM_005750	6.952
C10orf62	NM_001009997	5.4	0.0078				NST0000035946	NST0000035946	6.926
SMAD9	NM_005905	5.33	0.0363				TGM4	NM_003241	6.92
SPATA20	NM_022827	5.15	0.0088				WDR87	NST0000030386	6.909
C16orf78	NM_144602	5.14	0.0117				HSFX1	NM_016153	6.772

FIGURE 8.11 (Continued) DET and EET Lists from P and NP Groups from Different ARTs.

Gene	Accession	Fold change	p	Group	ART	Group	Gene	Accession	Value
APOE	NM_000041	28.27	0	Overexpressed in P group	Intracytoplasmic sperm injection	Exclusive expressed in P group	FSTL4	NM_015082	10.636
APOC1	NM_001645	27.9	0				TNMD	NM_022144	10.43
CFD	NM_001928	7.74	0.0067				LOC646808	XR_017339	9.793
CTSZ	NM_001336	6.85	0.015				C22orf26	NM_018280	9.756
HMOX1	NM_002133	6.58	0.012				CXorf34	NM_024917	9.446
FTL	NM_000146	6.35	0.0183				MBOAT4	AF359269	9.378
TGFBI	NM_000358	6.06	0.0157				UBQLN4	NM_020131	9.361
CTSL	NM_001912	5.93	0.0163				ALDOC	NM_005165	9.136
LGALS3	NM_002306	5.6	0.017				ANGPTL4	NM_139314	8.869
CD63	NM_001780	5.48	0.0183				SHFM3P1	AF174606	8.741
NST000003176E	NST0000031763	4.09	0.0456	Overexpressed in NP group		Exclusive expressed in NP group	DSG1	NM_001942	9.996
COX7B2	NM_130902	3.82	0.049				RPGR	NM_001023582	9.053
C19orf36	NM_001039846	3.8	0.0464				KLRC3	NM_007333	8.902
ANKRD7	NM_001077708	3.79	0.0467				CYP3A7	NM_000765	8.794
CDKN2D	NM_001800	3.7	0.0492				ERN2	NM_033266	8.744
							RP11-327P2.4	AK124707	8.67
							GFM1	NM_024996	8.658
							TMEM144	NM_018342	8.528
							INSM2	NM_032594	8.528
							C1orf74	NM_152485	8.474

FIGURE 8.11 (Continued) DET and EET Lists from P and NP Groups from Different ARTs. *Note:* We only provided the 10 most DET and the 10 EET with higher fluorescence value.

common in most physiological processes (i.e., response to stress, defense response, vacuole, membrane, plasma membrane, protein binding, signal transducer activity, or adenosine triphosphate [ATP] binding). The fact that gene lists obtained after sperm microarrays analysis do not show GO terms directly related to male reproduction or gamete generation as they should reveal the current widespread lack of knowledge about real sperm or male reproduction physiology (Figures 8.12 and 8.13).

GO analysis DET			
Intrauterine insemination	Biological processes	Immune system process	
		Immune response	
		Defense response	
		Response to stimulus	
		Inflammatory response	
	Cellular components	Integral to plasma membrane	
		Intrinsic to plasma membrane	
		Plasma membrane	
		Plasma membrane part	
		Lysosome	
	Molecular functions	IgG binding	
		Immunoglobulin binding	
		Protein binding	
		Molecular transducer activity	
		Signal transducer activity	
In vitro fertilization	Biological processes	Inflammatory response	
		Defense response	
		Response to wounding	
		Immune system process	
		Immune response	
	Cellular components	Extracellular region	
		Extracellular region part	
		Extracellular space	
		Lysosome	
		Lytic vacuole	
	Molecular functions	Cadmium ion binding	
		Copper ion binding	
		Cytokine activity	
		Protein binding	
		Chemokine activity	
Intracytoplasmic injection	Biological processes	Response to wounding	
		Inflammatory response	
		Negative regulation of hydrolase activity	
		Response to stress	
		Response to external stimulus	
	Cellular components	Extracellular region	
		Extracellular space	
		Lysosome	
		Lytic vacuole	
		Vacuole	
	Molecular functions	Cadmium ion binding	
		Kininogen binding	
		Copper ion binding	
		Cysteine-type endopeptidase activity	
		Enzyme inhibitor activity	

FIGURE 8.12 DET gene ontology analysis. *Note:* We only provided the 10 most statistically significant biological processes, cellular components, and molecular functions.

Technique	Category		Category	
Intrauterine insemination	Biological processes	Regulation of immune system process	Biological processes	Multicellular organismal process
		Positive regulation of immune system process		System development
		Regulation of immune response		Multicellular organismal development
		Leukocyte activation		Anatomical structure development
		Regulation of response to stimulus		System process
	Cellular components	Intrinsic to plasma membrane	Cellular components	Plasma membrane part
		Integral to plasma membrane		Extracellular region part
		Plasma membrane part		Extracellular region
		Plasma membrane		Cell junction
		Integral to membrane		Plasma membrane
	Molecular functions	GTPase regulator activity	Molecular functions	Glycosaminoglycan binding
		Enzyme regulator activity		Heparin binding
		Nucleoside-triphosphatase regulator activity		Hydro-lyase activity
		Metalloendopeptidase activity		Pattern binding
		Guanylate cyclase activity		Polysaccharide binding
In vitro fertilization	Biogical processes	Immune system process	Biogical processes	Multicellular organismal process
		Regulation of multicellular organismal process		Multicellular organismal development
		Positive regulation of cytokine production		System development
		Immune response		Developmental process
		Regulation of cytokine production		Cell surface receptor linked signal transduction
	Cellular components	Integral to plasma membrane	Cellular components	Plasma membrane part
		Intrinsic to plasma membrane		Plasma membrane
		Plasma membrane part		Extracellular region
		Cell surface		Extracellular region part
		Cytoplasmic part		Intrinsic to plasma membrane
	Molecular functions	Cytokine binding	Molecular functions	Signal transducer activity
		Cytokine receptor activity		Molecular transducer activity
		Transferase activity		Receptor activity
		Nucleotidyltransferase activity		Calcium ion binding
		Transferase activity, transferring glycosyl groups		Transmembrane receptor activity
Intracytoplasmic injection	Biogical processes	Organ development	Biogical processes	Organelle fission
		Vasculature development		M phase
		Blood vessel development		M phase of mitotic cell cycle
		Anatomical structure development		Mitosis
		Response to molecule of bacterial origin		Nuclear division
	Cellular components	Membrane	Cellular components	Membrane-bounded organelle
		Plasma membrane		Intracellular
		Plasma membrane part		Intracellular membrane-bounded organelle
		Membrane part		Intracellular part
		Insoluble fraction		Intracellular organelle
	Molecular functions	Transforming growth factor beta binding	Molecular functions	Ligase activity
		Binding		Catalytic activity
		Growth factor binding		Adenyl nucleotide binding
		Pyrophosphatase activity		Binding
		Hydrolase activity, acting on acid anhydrides		Nucleotidyltransferase activity

FIGURE 8.13 EET Gene ontology analysis. *Note:* We only provided the 10 most statistically significant biological processes, cellular components and molecular functions from both P and NP group.

DET genes	DET	Technique	EET	EET genes
ADA, ADAM10, B4GALT1, BCL6, BMF, CCL3, CCL4, CHST11, FCGRT, FOS, GRN, IGFBP7, IL8, LFNG, LY6E, MAFB, MBNL1, NFE2L2, NIPBL, NOTCH2, NR4A3, P2RY2, PI3, PROK2, RGS2, SKI, SLC2A14, SLC30A1, SP100, SP3, SPHK1, TAP1, TFEB, THBD, UBR2, VASP, ZFP41, ZMIZ1	**DET P**	**IUI**	**EET P**	ADAM28, APAF1, APBA3, APC, APOBEC3G, B4GALNT1, BCL10, CEP290, CUL3, CXXC4, DAZ2, ECE1, EDN2, EN1, EREG, ESR1, FANCA, FOXD2, FURIN, FUT10, GLRA1, GNAS, HCFC1, HESX1, HTR2B, ICMT, ITGA4, MACF1, MORC1, NCOA3, NOTCH1, OTOP1, PDPN, PDPN, PLXNA2, POU2F1, SP8, SPIN3, STAT3, STRA8, TAF4, TBX6, TDRD1, TGFB2, TMIE, UCN, USF1, USF2, UTP14C, VCAM1, WT1, WWP2, ZSCAN2
ALOX15B, CDO1, CFC1, CHEK1, CYLC2, GPX3, HOXB6	**DET NP**		**EET NP**	ACE2, ADAMTS1, ADCYAP1, AXIN2, BCL2L1, BCL2L10, BMP5, BMP7, C15orf2, CALCR, CDX2, CEP57, COL1A1, CSF1, DCN, DLC1, DLX5, FBN2, FGF2, FOXL2, FZD6, GDNF, GJA1, HOXA3, IFNB1, KIT, KLF1, LAMA1, LAMA3, LEP, LNPEP, LRP5, NDST1, NRG2, OR2H2, OSR2, PAQR7, PLAC1, POU2F3, POU4F3, PRDX3, PRLR, PRMT7, PSG1, PSG11, PSG2, PTCH1, SATB2, SEMA3C, SIX4, SLC34A2, SLIT2, SMAD6, SYT6, TBX5, TCF7L1, USP22, WDR33, WNT2B
ADM, CCL2, CCL3, CD44, CEBPB, CXCR4, FOS, GRN, HEXB, IL1 B, MAFB, RGS2, SPP1	**DET P**	**IVF**	**EET P**	ACVR1, ADAM10, ADAM28, ALDH1A2, AMOT, APOBEC3G, ARNT, BIRC3, BMP4, BMPR1 A, C5, CD28, CDYL, CHST11, CRH, CRTAP, CYP27B1, DLL1, DMRTA1, EIF2AK3, EPOR, ESX1, FGF4, FOXK1, HHEX, HOXB4, HOXD1, HTR2B, IFT52, IGFBP7, LNPEP, LY6E, MAFF, MAP3K1, MEN1, MKL2, NDST1, NLRP14, NPY5R, OCA2, OXTR, PAQR8, PAX1, PBX2, PDPN, PIK3CB, PLA2G4C, PRPF19, RAB23, RBM19, RLN1, SFRP1, SGPL1, SH2B3, SKI, SMARCA5, SOHLH1, SPIN1, SRD5A1, SYT6, TCF7L2, TDGF1, TGFB1, THOC2, TOP2A, TSC1, TSHZ3, TSNAX, TXNRD3, USP22, XDH, ZP2
CFC1, DNAH9, OAZ3, ODF1, SMAD9, SPANXA1, SPATA20, SPATA3, TNP2	**DET NP**		**EET NP**	AMH, APBA1, AXIN1, B4GALNT1, BCL10, CALCA, CAPN11, CCL5, CDY2A, CELSR1, CLIC5, COL1A1, CREB1, DKK1, DLK1, DLX5, DMC1, DNMT3L, ECE1, EDA2R, EDNRA, ELF3, ESR2, FBN2, FOXA2, FOXF1, FOXI1, FOXL2, FOXP3, FSHR, GAL3ST1, GLI3, HAND1, HOXA10, HOXB7, HOXC6, HSD11B2, ITGA1, KDR, KLF1, LAMA2, LIF, LRP6, MFGE8, MLL2, MSH4, NOG, NR6A1, ODF3, OSR1, PCSK1, PCYT1B, PDGFRA, PGAP1, PIWIL1, POU2F3, PSG11, PSG4, PSG7, PTCH1, PTGDR, RARG, REN, SEMA3C, SERPINB5, SHH, SOX10, SOX15, SOX2, SPATA2, SPINT1, STRA8, SUFU, TAC3, TBX6, TFAP2C, TGFB1I1, THOC6, TIMP4, TUBD1, TULP3, UCN, VEGFC, WNT2, WNT3A, XAB2, ZFP41

FIGURE 8.14 DET and EET previously described in reproduction-related GO terms. *(Continued)*

CXCR4 GRN SPP1	DET P	ICSI	EET P	AATF, ACOX1, ACVRL1, ADAM15, AFF4, AFP, ANG, AR, ARC, ARNT2, ASCL2, ASZ1, BAT1, BCL10, BMF, BMP2, C19orf20, CALCA, CATSPER4, CCL5, CRISP1, CXXC4, CYLC1, DIAPH2, DMRT2, ECE1, EDA2R, EFNA1, ELSPBP1, ENG, EOMES, EPAS1, ERCC2, ESX1, FAM50A, FNDC3A, FOXC1, FOXD2, FOXF1, GATM, GJA5, GLI3, GNA12, GNA13, HFE, HMGCR, HMX2, HOXA2, HOXB1, HOXB8, HSD11B2, HUS1, IGF2R, IGFBP7, ITGA4, ITGA8, KRT9, LEP, LMO4, LRP6, MACF1, MADCAM1, MBNL1, MGST1, MSX1, MSX2, MYF6, MYH9, NCOA3, NLE1, NRG2, P2RX1, PAX5, PDGFRB, PGF, PIWIL4, PLCG1, POU2F1, PPAP2B, PPARD, PRDM1, PRDX3, PRLHR, PTF1A, PTPN11, RBP4, RUVBL1, RXFP2, SEMA3C, SIX1, SKI, SMAD5, STAT5B, SYT8, TAC3, TACR1, TGFBR2, THOC1, TIMELESS, TOB2, TUBD1, UBP1, VEZT, WNT1, ZP4
ANKRD7	DET NP		EET NP	ACD, ADAM20, ADAMTS2, AGPAT6, ALDH1A2, AMBP, APBA1, ARFRP1, ATP6V0A2, AXIN2, BOLL, CCNB1, CEP57, CETN2, CHEK1, CHUK, CLEC4M, COQ7, CRTAP, CSRP2BP, CUBN, DAZL, DDX25, DLK1, DNAJA3, ELL3, FAM48A, FBXW4, FOXA1, FOXI1, FSHR, FUT10, FUT8, GAPDHS, GIP, GOPC, HERC4, HOXB5, HOXD9, HSF2, HSP90AB1, INHA, JAG2, KDR, LAMA5, LIG3, LRSAM1, MAFF, MAK, MAPK1, MKKS, MLL, MLL2, MORC1, MST1R, MTL5, NANOS3, NAT8B, NF2, NRG1, NSD1, OTOP1, OVOL1, PAPPA, PAX7, PBX2, PCDH12, PGR, PKDREJ, POLR1 B, PRKAR1 A, PRL, PRMT7, PROX1, PSG1, PSG4, PSG5, PSG7, PSMC3, PSMC4, PSME4, PVRL1, PYGO2, RAD23A, RARG, RASA1, RNF17, SCEL, SIAH1, SIX2, SLC22A16, SPAG6, SPIN2B, SPO11, SYCP3, TBP, TCF7L1, TDGF1, TERF2, TESK2, TH, THOC6, TNF, TRAF6, TUBGCP3, ZBTB16, ZP1, ZP3, ZPBP2

FIGURE 8.14 (Continued) DET and EET previously described in reproduction-related GO terms.

We also searched for those DET or EET that have been described in some GO terms related to reproduction, as we explained in the previous section (sperm molecular feature required in ARTs). Results of this systematic research are shown in Figure 8.14. Only a low percentage of our DET and EET has been previously described in a reproductive-related GO term reflecting, again, the insight that the male reproduction physiology remains largely unknown.

Given the differences between the expression profiles of sperm samples that achieve pregnancy and those of samples that do not, analyses of said profiles could be performed in clinics to complement basic sperm analysis. A prospective study of the clinical usefulness of such analyses to predict pregnancies is being conducted at our unit.

In summary, there is no perfect test available to define a sperm sample as optimal to achieve a pregnancy. Only basic sperm analysis is used to predict male fertility, given its ease of performance and the consensus reached by Andrology Laboratories. The existence of a perfect test could lead to the selection of sperm samples with the highest probability of success, thus diminishing risks and the number of medical procedures needed to achieve pregnancies. Microarray evidence indicates the existence of several markers with limited implications for male fertility. In view of our data, it seems that a complex test based on customized arrays evaluates the fingerprint and is the most adequate approach to develop a diagnostic tool; but for the moment, both the commercial tests and the clinical validation have not been developed yet.

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9

The Application of Genetic Tests in an Assisted Reproduction Unit: DNA Fragmentation

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Scientific Background of Sperm DNA Damage

One in six couples trying for a family faces fertility issues. This is a stark statistic facing many couples today. Assisted reproductive technology (ART) was first used in the late 1970s and since its advent, more and more infertile couples have pursued this treatment to have a family. Women undertaking ART undergo a battery of diagnostic tests; however, in nearly half of these couples, the male partner has problematic sperm,[1] yet male partners simply receive the semen analysis test. Since the 1980s, a growing body of research has highlighted the significance of sperm DNA in predicting successful ART outcomes.[2,3] As a result of this growing research, numerous DNA fragmentation tests have been tried and tested, with well-proven links between sperm DNA damage and all fertility outcomes from fertilization, embryo quality, pregnancy, and recurrent pregnancy loss. This suggests that sperm DNA quality plays a pivotal role in successful human reproduction.[4,5]

There are three common types of DNA damage that occur in sperm: 8-hydroxy-2′-deoxyguanosine (8OHdG) adducts, single-strand breaks (SSB), and double-strand breaks (DSB).[6] Oxidative stress from reactive oxygen species (ROS) including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) can generate various types of damage. Bases are oxidized (typically guanine), generating these adducts such as 8OHdG that with characterization will provide a more detailed understanding of DNA damage.[7,8]

DNA damage assays are widely used as sensitive toxicological tests in somatic cells and are often centered on monitoring the expression or DNA repair proteins in the nucleus. Unfortunately, sperm are highly modified in comparison with somatic cells, and measurements of DNA repair proteins are not an option. During spermatogenesis, sperm cells undergo histone replacement with the nuclear protein protamine in the testes. It is this protamine replacement that acts to stabilize and condense the sperm DNA, and as the sperm cell reaches maturity, the formation of disulfide bridges between these protamines in the epididymis further protects the DNA from damage.[9,10] As a result of this repackaging of sperm DNA, and significant morphological reorganization, sperm have very limited DNA repair machinery, leaving them susceptible to exogenous DNA-damaging agents. Their unique plasma membranes also make them vulnerable to these agents.[7] Additionally, testicular sperm are more susceptible to secondary DNA damage (during sperm transport), as it has been shown that ejaculated sperm have increased levels of DNA damage compared to sperm that have not traveled through the epididymis (Figure 9.1).[11]

Sperm DNA lesions cannot be repaired by the sperm itself but some can be corrected by the oocyte postfertilization. It is believed that the oocyte has limited repair capabilities; however, it is not yet known if both SSB and DSB can be repaired.[9] These breaks can potentially lead to mutations in the embryo, resulting in short- and long-term disease in progeny.[6] Causes of DNA damage include ROS due to insufficient antioxidant defense, release of endonucleases from apoptosis, and insufficient protamine binding. These increase with paternal age as well as poor lifestyle choices such as smoking, recreational drug use, obesity, and disease conditions such as diabetes.[10,12,13]

Sperm DNA quality is important at every stage of offspring development from fertilization onward. Recently, Simon et al.[14] reported compelling evidence of how embryonic development was influenced by paternal effects immediately following fertilization. Previously, it has been thought that the paternal

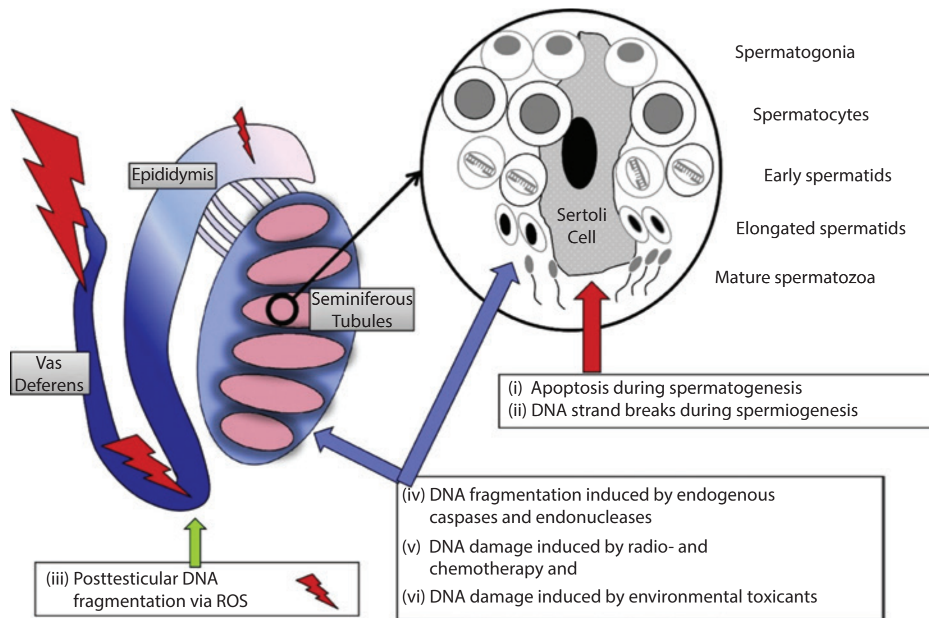


FIGURE 9.1 Mechanisms of DNA damage induced during spermatogenesis or transport from the testes. (Reprinted from Sakkas D, Alvarez JG, *Fertil Steril*, 93(4), 1027–36, 2010. With permission.)

genome was inactive until after the third cleavage stage. However, this study confirms that knowledge from previous reports (Simon et al.[15]) is outdated. The paternal genome appears to contribute to further embryo development as soon as the oocyte genome becomes transcriptionally active.[16] At the later stage of the reproductive process, we also need to consider the risks taken in assisting the creation of offspring with compromised sperm DNA quality, as there can be long-term effects on the health of the children.[6,13] This is important to couples and also to society at large with the increasing use of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) across Europe.

As an example of the effects of DNA damage in offspring, a higher incidence of hematological cancers such as leukemia was discovered in offspring conceived by men who smoked[6,17–21]; it was also found in animal studies that exposure to cancer therapeutic agents caused sperm DNA damage. Sperm DNA damage has been linked to a number of neurological and mental disorders such as Apert syndrome, schizophrenia, and achondroplasia but has also been associated with increased miscarriage rates following ICSI treatments.[6,22–24] As the quality of paternal DNA has such a wide-ranging impact on the health of the offspring, this points toward the need for routine DNA damage testing in sperm as best clinical practice. More research into the molecular understanding of the causes of DNA damage is also needed.

DNA Fragmentation Techniques

For clinical use, sperm DNA damage testing needs to be precise and repeatable, while remaining sensitive so the method is not limited by the number of sperm needed.[25–27] In comparison to the standard semen analysis testing the concentration, motility, and morphology, DNA fragmentation testing has a higher level of repeatability (reviewed by Lewis et al.[7]). In both infertile and fertile samples, the test should predict the outcome of pregnancy through measuring DNA strand breaks by lysing the cells and decondensing the DNA without additional damage caused.[28] There are currently a number of DNA fragmentation tests available, each utilizing unique investigational and analytic techniques. Each test investigates differing aspects of sperm chromatin status and DNA damage so they should not be clustered as the same test yielding the same result.[7,13,29]

Flow Cytometry

In assessing DNA damage, a number of DNA tests use flow cytometry for the electronic detection of fluorescence, possessing the high-throughput ability to assess multiple sperm parameters with high sensitivity.[27,30] Flow cytometry was developed in the 1970s for sorting cell populations using varied fluorescence probes in analyzing different properties of sperm. Through continuous development and optimization, this technique can be used in assessing sperm count, acrosomal reaction, viability, ROS, and chromatin status among others.[31] Although flow cytometry has the advantage of using high cell numbers, reducing time and labor, routine fertility clinics may not have access to the expensive facilities needed.[27] In analyzing sperm, the cells are labeled with a fluorescent tag detected at the "interrogation point" where the cells pass individually through a narrow point and are subsequently stimulated by a high-powered laser. Here, the fluorescence excitation is detected by multiple photodetectors and the signal amplified. This information is presented in the form of fluorescent intensity units using a cytogram.[32]

Fluorescence Microscopy

Fluorescence microscopy is an alternative analytical technique used to measure DNA fragmentation.[33] In comparison to flow cytometry, fluorescence microscopy can be more subjective with less sensitivity due to small undetectable changes in fluorescence or color from a dye or probe; however, there is a software available that can improve accuracy (see the section "Use of Flow Cytometry in Sperm DNA Fragmentation Tests" for further information).[27] Problems arise with the duration of observation, where bleaching can cause obscure results. However, recent developments improving both of these limitations allow more reliable results.[34]

Use of Flow Cytometry in Sperm DNA Fragmentation Tests

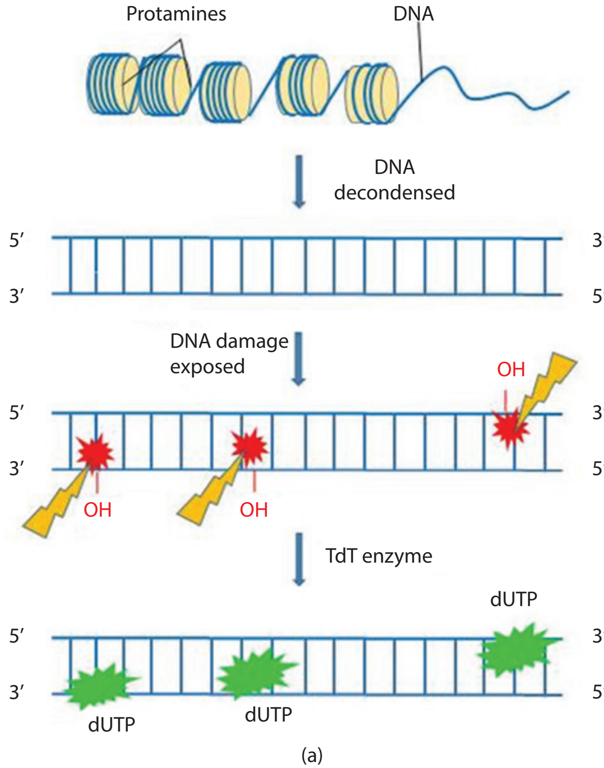
Four techniques are currently available in analysis of DNA damage in sperm using fluorescence microscopy or flow cytometry. The DNA fragmentation tests terminal transferase deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) and sperm chromatin structure assay (SCSA) use flow cytometry; however, fluorescence microscopy can also be used to analyze TUNEL samples. The alkaline Comet assay and sperm chromatin dispersion (SCD or Halo assay tests) use fluorescence microscopy for investigating sperm DNA. More details on these tests are discussed in the section "Terminal Transferase dUTP Nick-End Labeling," with the advantages and limitations of each discussed in the section "Comparison of DNA Fragmentation Tests."

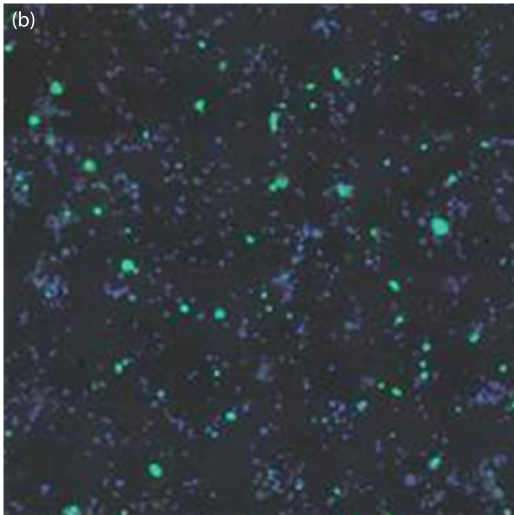
Terminal Transferase dUTP Nick-End Labeling

This assay can be used to investigate fragmented DNA in sperm measuring both single- and double-stranded DNA. This is one of the oldest tests that can be used to detect levels of apoptosis in somatic cells as well as to remove germ cells from the reproductive pool.[7,35] This method involves using modified nucleotides (dUTP) that are enzymatically added (using terminal deoxynucleotidyl transferase [TdT]) to 3′OH of broken DNA breaks, before the DNA "nicks" or ends are detected by fluorescence.[28,31,33,36] The fluorophore fluorescein isothiocyanate (FITC) is often used alongside propidium iodide (PI) or 4′,6-diamidino-2-phenylindole (DAPI) that stains all cells; for amplification, the anti-dUTP antibody can also be detected (Figure 9.2).[37] DNA damage can be assessed through flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California), although the equipment needed is expensive and requires the user to be highly trained; however, fluorescence microscopy is a viable alternative.[28,38] Figure 9.2 depicts stages of the TUNEL assay.

Sperm Chromatin Structure Assay

The SCSA detects a color change of acridine orange when sperm are exposed to acidic conditions. This measures the susceptibility of the whole semen population to DNA damage.[39] Chromatin with





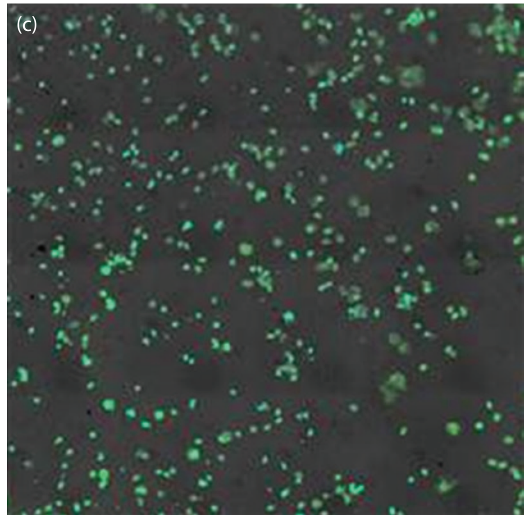


FIGURE 9.2 Use of fluorescence in TUNEL. (a) Schematic diagram of the TUNEL assay. The enzyme TdT is used to enzymatically bind dUTP to DNA breaks, which can be detected by flow cytometry. Fluorescence microscopy images of TUNEL. The green represents FITC and the counterstain used in blue is DAPI. (b) Example of low DNA damage. (c) Example of high DNA damage. (TUNEL, terminal transferase dUTP nick-end labeling; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; dUTP, deoxyuridine triphosphate; TdT, terminal deoxynucleotidyl transferase.)

strand breaks in an acidic environment will denature due to reduced stability, indicating DNA strand breaks. The stain acridine orange can then be used to differentiate between native DNA (fluorescent green) and denatured DNA (fluorescent yellow/red) (Figure 9.3) and measured using FACSCalibur flow cytometer. Software SCSASoft (SCSA Diagnostics, Brookings, South Dakota)[40–42] can be used where populations with low DNA damage (green) and moderate-to-high DNA damage (yellow/red)

are analyzed and from this the DNA fragmentation index (DFI) is calculated (Figure 9.4), that is, the percentage of the sperm population with moderate-to-high DNA damage (Figure 9.5). Sperm with high levels of green fluorescence have high DNA stainability (%HDS), simultaneously, %DFI is calculated by measuring the ratio of red to total (both red and green) fluorescent intensity used to form a DFI frequency histogram.[43,44]

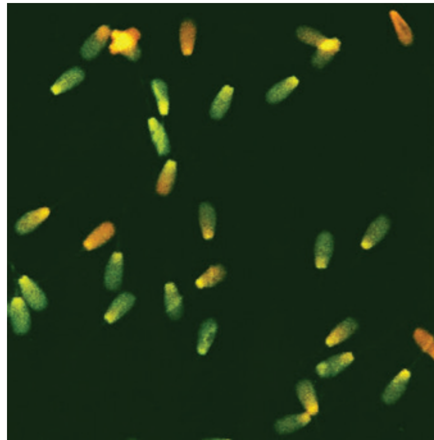


FIGURE 9.3 Acridine orange staining. Here different amounts of red and green fluorescence are detected depending on the DNA strand breaks. Events with more green show sperm without DNA damage, whereas orange to red cells are classified as having DNA damage.

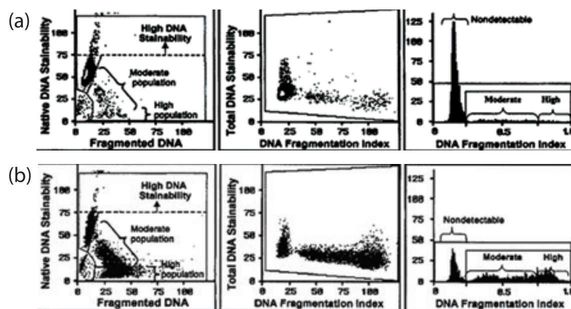


FIGURE 9.4 Example of SCSA data. (a) Undamaged DNA leading to pregnancy; (b) damaged DNA damage leading to no pregnancy. In the top panel, most of the sperm are in the low damage category, whereas in the lower panel, the majority of sperm have either moderate or high damage. Left panel: 5000 sperm that are plotted on a scatter diagram identifying three levels of sperm DNA integrity. The *Y* axis plots green fluorescence, and the *X* axis plots red fluorescence. Middle panel: Raw data in the left panel converted by the software, so the angled normal data are converted to a vertical pattern. Right panel: A frequency histogram of data from the middle panel, gating the data into three categories (normal, moderate DFI, and high DFI). To calculate total %DFI = moderate + high DFI. (DFI, DNA fragmentation index; SCSA, sperm chromatin structure assay.)

$$\text{DFI (DNA fragmentation Index)} = \frac{\text{Red fluorescence}}{\text{Total flurorescence (Red + Green)}}$$

FIGURE 9.5 The calculation of DNA fragmentation index (DFI); a measure of DNA damage.

Sperm Chromatin Dispersion

The SCD (or Halo) assay is a relatively new technique, testing for chromatin dispersion. It is the simplest technique involving the use of fluorescence microscopy (recently modified to use bright-field microscopy) to estimate the amount of DNA damage by looking at the formation of a halo.[45,46]

As with the SCSA, SCD involves acid denaturation of DNA and removal of nuclear proteins by dithiothreitol (DTT) treatment. For microscopy, a number of nuclear stains can be used, such as DAPI and Wright's stain, which are added before samples are scored with a minimum of 500 sperm under ×100 objective of the microscope.[36,45] The test works under the principle that relaxed loops of DNA, which are attached to the central core of the nucleoid, disperse in an agarose gel to produce a halo.[45,47,48] This indicates little or no DNA fragmentation. If the DNA is fragmented, the halo size will be reduced in relation to the level of DNA damage or no halo will be present (Figure 9.6).[49]

Alkaline Comet Assay

The alkaline Comet assay (originally known as single-cell gel electrophoresis) is a second-generation DNA fragmentation test. This test quantifies DNA damage per sperm using single-cell gel electrophoresis followed by fluorescence microscopy.[50] It is the only test where actual DNA damage is quantified at the individual cell level, which is particularly important when investigating heterogeneous cell populations.[6,51] The sperm are embedded in agarose on a glass slide and treated with lysis buffer. Additional treatment is needed to remove protamines and histones as the compact structure of DNA prevents it migrating during electrophoresis. These can include Triton X-100, dimethyl sulfoxide (DMSO), DTT, lithium 3,5-diiodosalicylate (LIS), and/or proteinase K.[15,51,52] The migration of damaged fragments in electrophoresis causes a "Comet" effect where the distance the fragments of damaged DNA migrate into the Comet tail is dependent on their size. They can be scored by fluorescence microscopy (using ethidium bromide or SYBR Green for example) using dedicated imaging software.[36] There are numerous software packages available for analyzing the alkaline Comet, including free software such as ImageJ and CometScore, as well as those commercially available such as Komet 5.5 (Andor BioImaging, Belfast, United Kingdom), MetaSystems automated imaging (MetaSystems Group, Newton, Massachusetts), and Comet Assay IV (Perceptive Instruments, England, United Kingdom).[53] There are three commonly used parameters to measure DNA damage by the alkaline Comet. The first is the tail length (from the leading edge of the head), the second is the tail DNA (percentage found in the tail compared to the head), and finally the olive tail moment (OTM), which can be defined as the tail DNA multiplied by the distance between the means of tail and head fluorescence.[6]

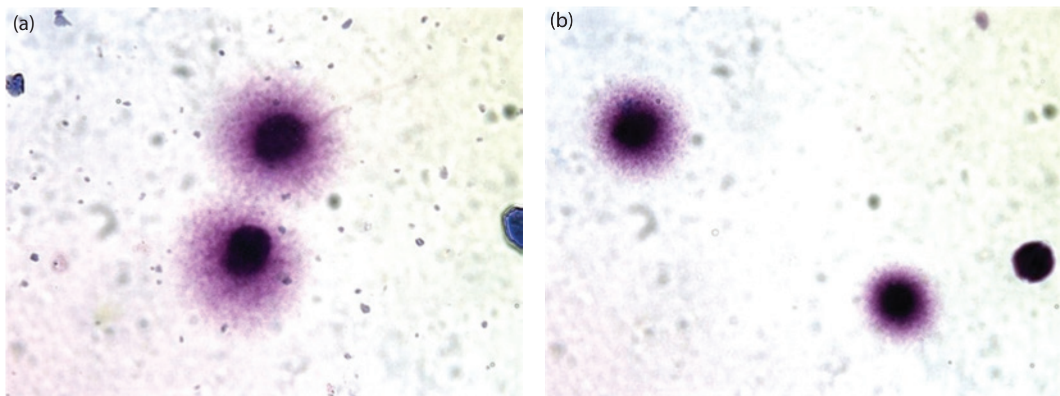


FIGURE 9.6 Example of the sperm chromatin dispersion test showing various sized halos with (a) large halo, and (b), medium halos, small halo, and no halo (left to right). Fragmented DNA is indicated by a small halo or its complete absence.

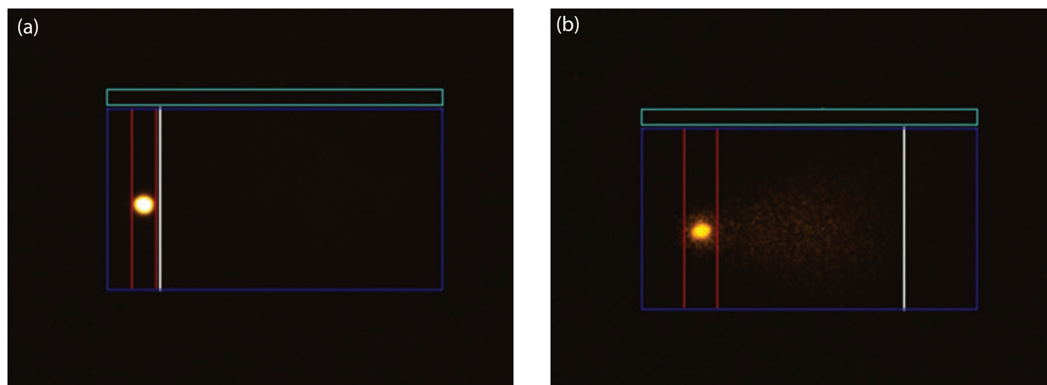


FIGURE 9.7 Use of Comet software. (a) Low DNA and (b) high DNA. DNA is stained with ethidium bromide and comets are selected with the dark blue box. Head intensity is measured between the red bars. Tail intensity is measured from the second red bar to the white bar. The light blue box on top measures background. The software measures various parameters for calculating DNA damage.

Clinical Evidence of Relationships between Sperm DNA Damage and ART Outcomes

The commonly used DNA damage tests described in the section "DNA Fragmentation Techniques" have been compared in assessing their clinical use in relation to ART outcomes. It is becoming more apparent that DNA testing is essential since high DNA damage increases risk of pregnancy loss, regardless of which test is used (Comet, TUNEL, or SCSA) (odds ratio:2.5; 95% confidence interval [CI] 1.5–4.0).[54] Further, with pregnancy rates for IVF and ICSI remaining the same year after year, more research needs to go into investigating anomalies in the gamete of the male partner, and sperm DNA tests show the most promise of any novel tests to date.[55]

Many studies have sought correlations between traditional semen variables tested in clinics and DNA fragmentation but found little connection. This is not surprising as DNA tests assess sperm quality at a molecular level, whereas a semen analysis focuses on gross light microscopic parameters. The semen analysis is now recognized as having limited value in the diagnosis of male infertility and also prediction of ART outcomes.[28,56,57] For example, the study by Simon et al.[56] using the alkaline Comet assay has demonstrated that 68% of IVF patients were normozoospermic (by the World Health Organization [WHO] criteria) yet almost half had high DNA damage of above 48%. When comparing ICSI patients, no correlation was found with the semen analysis parameters.[56] Similarly, Meseguer et al.[58] found that ~20% normozoospermic men had DNA damage as assessed by SCD. There are some conflicting results showing relationships between motility and DNA damage.[59] The discrepancies may be due to differing protocols.[28,56] Results from IVF and ICSI cannot be directly compared due to differences in exposure to secondary DNA damage; for example, in IVF, sperm are left exposed overnight to culture media and potential for secondary DNA damage (from ROS production).[15,27] As previously discussed, high levels of ROS cause DNA damage due to excess residual cytoplasm, occasionally found even in fertile men.[13]

Of the four sperm DNA damage tests described, only SCSA and the alkaline Comet assay have established thresholds for clinical use in determining ART outcomes. The TUNEL has limited use in clinics as it has not been standardized for clinical use. Sharma et al.[60] reported a cutoff of 19.25% of normal healthy donors, with the hope that other centers could use this value to develop their own; however, there is still much variation in protocol between laboratories. Simon et al.[56] listed some of the thresholds set, for example, 4%,[61] 10%,[62] 15%,[63] 20%,[64–66] and 35%.[67] These thresholds however only distinguish between fertile and infertile men, unlike the alkaline Comet assay and SCSA that have set thresholds for low, medium, and high DNA damage, which are used clinically to direct patient treatment.

In a recent study (Simon et al.[16]), the alkaline Comet assay was reported to have the greatest sensitivity, detecting DNA damage in 73% of sperm in comparison to 13% and 15% in the same samples with SCSA and TUNEL, respectively. However, when comparing the tests, TUNEL correlated with the alkaline Comet assay, but not with SCSA. The alkaline Comet assay and TUNEL have shown correlation

between DNA damage and fertilization rates, as well as implantation rates.[29] Yet it was also found that TUNEL and SCSA significantly correlate with detection rates, which is surprising since they are clearly measuring different aspects of gamete quality due to variation in test conditions.[33]

Research by Pérez-Cerezales et al.[29] reported no correlation between SCSA (DFI) and alkaline Comet tail DNA, although both measure DNA fragmentation and are the most sensitive tests. Although SCSA is currently the most prominently used DNA damage test by clinics, the alkaline Comet assay has sensitivity and specificity of 85% and 92%, respectively; yet SCSA has a sensitivity of 60% and specificity of 88%.[66]

The SCD test has the least clinical data to support its use in a routine ART setting. Studies have shown no significant associations using ejaculated spermatozoa[49,68,69] or testicular sperm.[45] However, in an interesting study by Meseguer et al.,[70] sperm DNA damage as measured by the SCD assay had a negative impact on pregnancy if the oocytes were from infertile women (n = 98) but not if donor oocytes (n = 112) were used. This result was found with a mixture of IVF and ICSI treatments, and it is surprising in that no DNA test to date has a predictive power for ICSI outcomes. In the most recent study (Anifandis et al.[71]), sperm DNA damage was associated neither with embryological data nor with pregnancy rates.

Clinical Interpretation of DNA Fragmentation Tests: Need for Agreed Thresholds

Through research into various DNA fragmentation tests (see the section "Clinical Evidence of Relationships between Sperm DNA Damage and ART Outcomes" with clinical evidence), clinical thresholds need to be set so that clinics using the tests will be able to interpret them easily. For the alkaline Comet, SCSA, and TUNEL tests, the thresholds decided are shown in Figure 9.8, with different choices in ART treatment suggested, depending on the amount of damage. However, for TUNEL, this is an average of 20% of various thresholds discussed in the section "Clinical Evidence of Relationships between Sperm DNA Damage and ART Outcomes," which is only a threshold for infertility. This does not indicate whether IVF or ICSI is the correct treatment pathway if above this threshold as the data are inconclusive about TUNEL and ART outcomes. Figure 9.8 shows a suggested patient pathway dependent on different thresholds of sperm DNA damage. With this information and as data accumulate, clinics can help patients further inform their treatment choices and make bespoke decisions for individual couples. As new sperm are produced every 72 days, changes in lifestyle may also be considered if DNA damage is high so men may reduce their levels of sperm DNA damage, and thus potentially increase their probability of success.

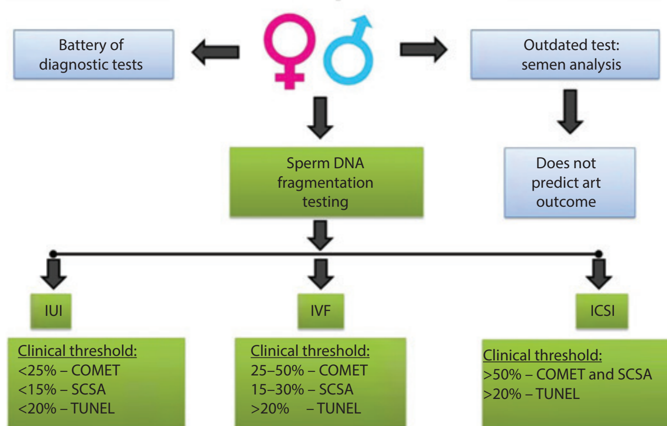


FIGURE 9.8 Thresholds for Comet, SCSA, and TUNEL. Comet and SCSA are more commonly used in the clinics due to their threshold for low, medium, and high DNA damage, and therefore can indicate which ART treatment should be suggested to the patient. However, TUNEL has only one threshold for male infertility. This figure shows the average of varied thresholds discussed in the text. (TUNEL, terminal transferase dUTP nick-end labeling; SCSA, sperm chromatin structure assay; dUTP, deoxyuridine triphosphate.) (With kind permission from Springer Science+Business Media: *Genetic Damage in Human Spermatozoa,* Sperm DNA fragmentation and base oxidation, 2014, pp. 103–16, Lewis SE; Sergerie M et al., *Hum Reprod*, 20(12), 3446–51, 2005; Ribas-Maynou J et al., *Andrology*, 1(5), 715–22, 2013.)

Simon et al. found that when assessing the outcome of IVF using the alkaline Comet assay, when DNA damage was above 50%, the live birth rate was reduced from 26.9% to 13.1% compared to ICSI treatment, which had shown a smaller decline in birth rate of 30.2% and 20.4% when DNA damage was beyond 50%.[15,27]

The impact of DNA damage on the outcome of intrauterine insemination (IUI), IVF, and ICSI was investigated using SCSA. Results from IUI had shown that the odds ratio of pregnancy was significantly lower with DFI > 30%. No significant difference was reported in pregnancy rates between low and high DFI groups (using the threshold of 30%) in IVF and ICSI. Yet, when comparing the odds ratio of pregnancy (2.25, 95% CI 1.10–4.60), ICSI was significantly better when the DFI was above 30%. This result could be due to the different culture environments between the two techniques.[27,72]

If the patient has high DNA damage (>50% using both alkaline Comet assay and SCSA), ICSI is suggested. However, if the male partner has intermediate sperm damage (25%–50% alkaline Comet, 15%–30% SCSA), IVF is suggested. This is beneficial for two reasons. First, IVF is cheaper than ICSI; additionally, in some cultures, couples have ethical and religious concerns about the use of ICSI.

Costs and Clinical Use

Although 40% infertility issues among couples are due to male issues, semen analysis is currently the only routinely used test in the clinic to diagnose male infertility.[73] As sperm DNA damage has been shown to correlate inversely with ART success, it would be useful if DNA testing became a routine test alongside semen analysis to provide a more informed choice of ART treatment.[74] More than 5 million children have been born through ART treatment worldwide, so ART is now a societal tool to increase national birth rates. Thus, it is important to make the most informed decisions for healthy offspring when deciding on treatment for significant sections of the European population. It is important for clinics to give not only safe clinical practice but also to assist couple ethically in getting pregnant with relative expediency, limiting costs and prioritizing the potential child's health across the European continent.[75]

The current costs of these DNA fragmentation tests are a small portion of what is spent on ART treatment (roughly €176–€480; see Table 9.3). In comparison, IVF and ICSI treatments are significantly more expensive (see Table 9.1). With this in mind, how can DNA fragmentation tests offset the cost of IVF/ICSI treatments and what is the benefit of DNA fragmentation tests in the patient pathway?

It is clear from the clinical evidence presented above that DNA fragmentation analysis is a powerful tool when selecting the best choice of ART for each couple. The European Society of Human Reproduction and Embryology (ESHRE) guidelines suggest ICSI should be used only in the event of severe sperm abnormalities.[76] However, in some countries, ICSI is used exclusively or predominantly, irrespective of semen profiles. Although there is discussion as to whether this constitutes best clinical practice, ICSI should be considered for couples with idiopathic infertility and following a failed cycle of IVF. However, with current treatment pathways, sperm DNA quality is not assessed and therefore not considered to be a severe sperm abnormality. This requires revision given the plethora of studies supporting its inclusion and the inadequacy of conventional semen analysis. Studies involving the SCSA and alkaline Comet assay have shown that those patients with high DNA damage (>30% DFI for SCSA and >50% for alkaline Comet assay) had a 1.3-fold increase

TABLE 9.1

Average Range of Assisted Reproductive Technology Costs (Collated from Online Fertility Clinics)

Region	IVF (€)	ICSI (€)
United Kingdom	3000–5000	4000–7000
United Kingdom (including London)	3000–7000	4000–7000
Spain	3000–6000	4000–6000
Czech Republic	2000–6000	2000–8000

Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic injection.

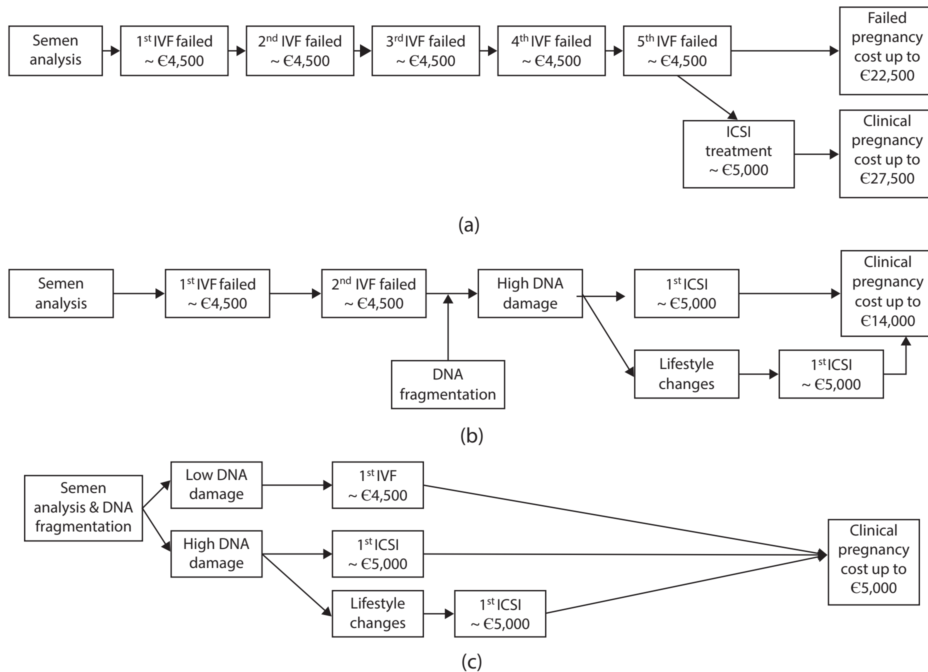


FIGURE 9.9 Various treatment pathways. The costs involved with and without DNA fragmentation tests affecting the number of cycles and type of assisted reproductive technology (ART) treatment based on average prices in Spain (collated from prices online from fertility clinics). (a) Model 1. Diagnosis based on Semen analysis only (b) Model 2. Sperm DNA testing to identify cause of ART failure and (c) Model 3. Sperm DNA testing as routine male work up.

in a successful clinical pregnancy with ICSI treatment compared to IVF treatment.[15,72] This highlights the need for DNA fragmentation testing as an important adjunct to the male workup when selecting treatment modalities.

There exist three potential models (Figure 9.9) to assess the cost benefit of DNA fragmentation tests in the patient pathway:

1. Semen analysis only with no DNA fragmentation test
2. Sperm DNA fragmentation test only following repeated ART failure
3. Routine sperm DNA fragmentation test as part of routine male workup prior to ART

Model 1 shows couples undergoing numerous cycles of IVF, with no DNA fragmentation testing. Although success rates vary country to country, analysis of world data reported an average delivery rate per cycle of 21%.[77] This would result in patients undergoing up to five IVF cycles before they potentially achieve a live birth or even consider ICSI as a form of treatment. If we look at Table 9.1, which shows comparative costs of IVF and ICSI cycles in the United Kingdom, Spain, and Czech Republic, we can calculate that patients would have to spend up to €22,500. This is a huge financial burden for couples but also those institutions that are publically funded, such as the National Health Service (NHS) in the United Kingdom. Repeated failed cycles not only come at a financial cost, but also at a personal/emotional cost for the couple. Most couples will experience a level of emotional distress during their treatment such as guilt, fear, stress, anxiety, and depression, which puts enormous pressure on relationships.[78,79] There is also a physical impact of repeated hormone treatments and invasive procedures, and a culmination of these financial and personal cost leads to 23% patients prematurely stopping ART treatment because of this perceived burden of treatment.[80] For all these reasons, it is essential to reduce the time and cost of ART treatments.

If we consider the models where DNA fragmentation is utilized, we can see its potential benefits in terms of these financial, physical, and emotional costs. Often in the late stages of test uptake, it can be used as a diagnostic tool to explain the causes of repeated failed cycles (as seen in model 2).

If a diagnosis of high DNA damage was made, patients could be placed in the optimal treatment pathway, ICSI, improving their chances of success. This has a knock-on effect in a number of respects. First, the diagnosis provides patients with a reason for their failed cycles and also opens the option of lifestyle changes and antioxidant treatment. There is evidence to show that men who smoke, drink, take recreational drugs, and are overweight have higher levels of sperm DNA damage.[2] This may be treated by antioxidant supplements; however, there is conflicting evidence on this issue and further research is required.[81–83]

Second, it saves couples time, particularly if women are over the age of 35 where their fertility decreases dramatically.[55] Third, it can reduce cost for the patient. The appropriate selection of ICSI treatment may reduce the number of cycles required to achieve the same success as IVF and this will reduce the overall cost for the couple. Finally, a reduction in unsuccessful treatments will also remove some of the emotional and physical burden of failed ART.

Ideally, the full benefit of DNA fragmentation testing would be realized if it was incorporated in the initial workup, along with semen analysis, as shown in model 3. The benefits outlined in model 2 would be amplified as high DNA damage would be identified earlier and patient care optimized.

Due to the high costs and increasing infertility, the WHO has recommended that there should be more public funding for ART as they consider it an increasing global health problem.[75] With this in mind, DNA fragmentation testing provides a more efficient way for clinicians to choose ART treatment and addresses ESHRE guidelines of requirements for ICSI treatment.[76] If there is to be more public expenditure on ART, there needs to be targeted treatments based on individual couples to maximize the impact of that expenditure. DNA fragmentation tests can provide an additional tool to optimize patient treatment. This optimization also results in reduced emotional and psychological costs. In 2013, a study revealed that patients received adequate psychosocial care and this would translate into a 15% increase in pregnancy rates, and as it has been described, DNA fragmentation testing can go a long way to help improve the psychosocial care of patients.[84]

Methods to Improve Sperm DNA Fragmentation Testing

Comparison of DNA Fragmentation Tests

There is still debate as to which test is best for clinical use. The first step needs to be standardization of protocols through international collaborations to establish clinical thresholds.[6,41] However, as with other molecular tests, outsourcing will probably be the most common route for use, due to the technical knowledge and training needed by the operator and the expense of the setup and use of sensitive assays for DNA damage when using flow cytometry and microscopy. Some clinics are already outsourcing to companies to perform SCSA and alkaline Comet assays as they have standard thresholds in predicting DNA damage and IVF success.[74] Although TUNEL can be conducted commercially, it is currently not common practice due to it's limitations. See Table 9.2 for details on positive attributes, limitations, and improvements recently reported for each test.

Customized Sperm Selection

Although researchers have managed to overcome some of the limitations in Table 9.2, none of these methods are entirely suitable for use in a clinic. Novel techniques are currently being investigated for sperm selection in the clinic. Sperm have been prepared using density gradient centrifugation (DGC) for more than 15 years as a means of isolating morphologically normal sperm. Others have previously shown how this technique improves DNA/chromatin integrity; however, this was investigated further by Bungum et al.[43] and Simon et al.[88] who found that when comparing SCSA or alkaline Comet analysis on sperm prepared by DGC, compared to native semen, the main conclusion was that isolation does not improve the predictive sensitivity of either test. Rather, it lessens its power.[43] How DGC effects DNA integrity was tested using TUNEL, where it was discovered that this technique did not select for sperm with higher double-strand DNA integrity.[89]

To improve ART success, the use of Magnetic Activated Cell Sorting (MACS) helps to separate apoptotic from non-apoptotic sperm cells. This method uses Annexin V microbeads, which bind to apoptotic sperm

TABLE 9.2

Advantages, Limitations, and Improvements Recently Reported for DNA Fragmentation Tests

	TUNEL	SCSA	Comet	SCD
Advantages	- Measures DNA single- and double-strand breaks directly[28,33,85] - Widely used in Italy and Spain - Tests to date show its ability to predict ART outcomes	- Standardized protocols for all labs using it - Many studies show its reproducibility between labs and its benefit in ART for couples with high DNA damage or idiopathic infertility	- Directly measures DNA damage in histone- and protamine-associated chromatin - Requires only 5000 sperm - Measures DNA damage of each individual sperm[15] - Generates numerous parameters of DNA damage	- Simple, fast, and inexpensive - Requires a bright-field microscope or fluorescence microscope[86]
Limitations	- Lacks standardized protocoal; needs further optimization for IVF[25] - Needs further data correlating it with ART outcomes [64,65]	- Unsuitable for samples with low sperm counts	- Labor intensive - Lacks standardized protocols, therefore currently needs to be outsourced[28]	- Qualitative, not quantitative as the size of the halo is only measured as low, medium, high, or absent - Issues with bleaching of the fluorescence - Not yet been proven that it measures DNA damage[36]
Improvements recently reported	- Lysis with DTT increases sensitivity as the DTT helps the chromatin to relax for access to "nicks"[33,50] - Use of dual staining, viability of the sperm measured alongside DNA damage; this uses live/dead fixable dead cell stain (which is far red)[33] - Propidium iodide (PI) improved the assay where brighter and dimmer populations were measured; it was discovered that the brighter fraction adds new information, as its predictive power was independent of age and semen quality. This variation of the assay gives low intraindividual variability of fragmented DNA while maintaining sperm viability, increasing the clinical relevance of the TUNEL[33,87]	- Standardized protocol has not been modified recently	- A modified alkaline Comet assay has been developed where the enzyme formamidopyrimidine DNA glycosylase (FPG) (derived from *Escherichia coli*) is used, converting base adducts to strand breaks[29,52]	- None known to date

Abbreviations: TUNEL, terminal transferase dUTP nick-end labeling; SCSA, sperm chromatin structure assay; SCD, sperm chromatin dispersion; ART, assisted reproductive therapy; DTT, dithiothreitol; dUTP, deoxyuridine triphosphate.

cells with externalized phospatidylserine (PS).[90] Meta-analysis of this technique concluded that MACS is a safe and efficient method that may help improve ART outcomes. Gil et al.[91] showed that pregnancy rates had improved; however, the rates of implantation and miscarriage did not change. Although this technique separates cells regardless of DNA fragmentation, it is important to reduce apoptotic sperm used in ART.[91] Yet, Romany et al.[92] showed that there was no improvement in reproductive outcome for ICSI using Annexin V selection; therefore, more research is needed for establishing new MACS protocols.

ICSI does not select for sperm with aneuploidy; however, a new technique is being tested where hyaluronic acid (HA) binding (found on the sperm membrane) can be used in isolating abnormal sperm, improving the chances of pregnancy after ICSI.[90] Worrilow et al.[92] termed the selection process physiologic ICSI (PICSI), where further research has found the binding between sperm and HA can facilitate the selection of individual sperm that have improved characteristics such as increased nuclear integrity, increased developmental and cytoplasmic maturity, and functional competence.[93,94]

Test Availability and Cost

Costs were found either on the company's website or via quotes by contacting the company directly (Table 9.3).

TABLE 9.3

DNA Fragmentation Test Availability and Contact Information

	Address	Cost (€)	Contact Number, Email, and Website
SCSA			
SCSA Diagnostics	302 6th Street West, Suite B, Brookings, South Dakota 57006	280	+1 866 219 1338 scsa@scsatest.com https://www.scsadiagnostics.com/
Biomnis	Biomnis, Lyon Laboratory, 17/19 Avenue, Tony Garnier, 69007 Lyon, France	176	+353 1 295 8545 sales@biomnis.ie http://www.biomnis.ie/index.aspx
SPZLab	SPZ Lab A/S, Fruebjergvej 3, 2100 Copenhagen OE, Denmark	380	+450 39179784 info@spzlab.com http://www.spzlab.com/
Alkaline Comet			
SpermComet Ltd	SpermComet Ltd, Queen's University Belfast, Institute of Pathology, Grosvenor Road, Belfast, BT12 6BJ, UK	290	+44(0) 28 9023 8915 info@spermcomet.com http://www.spermcomet.com/
SCD			
Halosperm G2 kit from Halotech	Perform in house	508 (for 10 tests)	(+34) 91 279 69 50 info@halotech.es http://www.halotechdna.com/productos/halosperm/
TUNEL			
Instituto Bernabeu	Avda. Albufereta, 3103016 Alicante, Spain	254	+34 965 50 40 00 info@institutobernabeu.com https://www.institutobernabeu.com/en/
FivMadrid	Marques de Urquijo 26, 1D Juan Alvarez Mendizabal, 74, 28008 Madrid, Spain	350	+34 915 616 616 consultavalladolid@fivmadrid.es http://fivmadrid.es/
CREA	Carrer de Sant Martí, 4, 46003, Valencia, Spain	480	+34 963 52 59 42 info@creavalencia.com http://www.creavalencia.com/EN/

Conclusion

As sperm DNA damage has an inverse correlation with ART success, there is ever-increasing support in the literature for routine sperm DNA fragmentation testing (using SCSA, TUNEL, and alkaline Comet assay). These tests help couples make informed decisions about their ART treatment pathway, reducing the financial and emotional burdens and increasing clinic success rates. Additionally, large studies are needed with standardized protocols to reduce interlaboratory variation to strengthen the evidence base for sperm DNA testing for clinical use.

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Conflict of Interest

Sheena E.M. Lewis is the managing director of SpermComet Ltd, the University spin-off company marketing the SpermComet test.

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10

The Application of Genetic Tests in an Assisted Reproduction Unit: DNA Methylation Defects

Cristina Camprubí and Joan Blanco

Epigenetics: DNA Methylation

Epigenetics refers to heritable changes in gene expression that occur without modifications at the DNA sequence level. Changes are regulated by DNA methylation at the 5-carbon of cytosine (5-methylcytosine [5mC]), posttranslational modifications in the N-terminal amino acids of histones, and the presence of noncoding RNAs. These epigenetic mechanisms act together to control chromatin structure to confer cell-specific gene expression. Recently, other types of cytosine modifications, including 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC),[1,2] have been described.

DNA methylation occurs at cytosine residues that are followed by a guanine (CpG dinucleotide). The addition of methyl groups is catalyzed by DNA methyltransferases (DNMT) that use S-adenosylmethionine (SAM) as a donor of methyl groups. Four active DNMTs are known in mammals: DNMT1, DNMT3A, DNMT3B, and DNMT3L.[3] DNMT1 is mainly associated with methylation maintenance in somatic cells because it recognizes the hemimethylated CpGs and catalyzes the methylation of the new chain in DNA replication and repair. DNMT3A and DNMT3B participate in de novo methylation during development leading to the establishment of new methylation patterns. Finally, DNMT3L is a cofactor that interacts with DNMT3A and DNMT3B in germ cells enhancing their activity.[4]

DNA methylation is an epigenetic mechanism involved in the regulation of many biological processes including cell-specific gene expression, genomic imprinting, and genomic stability:

- *Cell-specific gene expression:* The relationship between DNA methylation and gene expression depends on the methylated part of the transcription unit.[5] Methylation at promoter regions correlates with decrease expression, whereas body gene methylation has been associated to gene expression and alternative splicing.[5] Less is known about the methylation influence of the CpG located at kilobases of the untranslated regions (UTRs). The specific DNA methylation pattern of every gene meshed with the presence of specific transcription factors drives cells to differentiate and promote their specific functions.
- *Genomic imprinting:* The biological process whereby a gene or cluster of genes is marked by repressive or active epigenetic modifications according to its parental origin is known as genomic imprinting. This process regulates monoallelic expression of approximately 100 genes in humans, so that only the unmethylated maternal or paternal allele is expressed. Imprinted genes are distributed in different chromosomes and their functions are critical for proper neurological, embryonic, and extra-embryonic tissues development.[6] Importantly, the epigenetic marks that control the expression of imprinted genes are common in all cell types; this mechanism ensures its monoallelic expression in the cells in which they are expressed.
- *Genomic stability:* Proper DNA methylation of centromere and telomere regions is essential for chromosome dynamics and stability. Abnormal methylation of centromeric and telomeric regions has been associated with chromosome instability leading to cancer.[7,8]

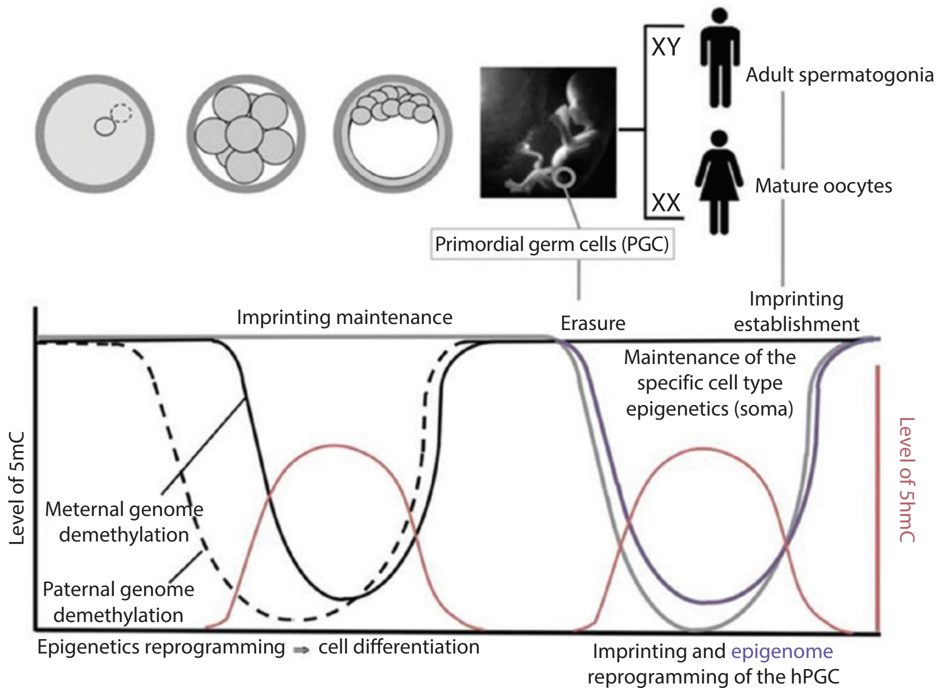

FIGURE 10.1 Epigenetic reprogramming during development. Black line, global genome methylation levels; gray line, methylation levels of the genes regulated by genomic imprinting; purple line, primordial germ cells' (PGCs) global genome methylation levels; red line, hydroxymethylation levels.

There are three periods of global resetting of DNA methylation during a man's life. The first occurs in the preimplantation embryo, the second in primordial germ cells (PGCs), and the third at spermatogenesis (Figure 10.1). At the end of the process, the sperm methylome has a very specific pattern that is closely related to the changes that take place during these three reprogramming events:

- *Preimplantation embryo:* The epigenetic patterns of gametes are erased during the early stages of embryonic development. As a result, cells resulting from the first mitotic divisions are totipotent and may lead to both embryonic and extra-embryonic components. At the blastocyst stage, epigenetic patterns of pluripotent cells that define the trophectoderm and the inner cell mass begin to emerge. These cells can result in any of the three embryonic lineages (endoderm, ectoderm, and mesoderm) and so, gradually during development, the epigenetic patterns that will define cell types, tissues, and organs emerge.

The demethylation of maternal and paternal genomes during early embryonic development is asynchronous. The demethylation of the paternal genome is an active process regulated by the action of ten-eleven translocation (TET) proteins through oxidative reactions.[9] 5mC oxidation generates 5hmC, which is subsequently transformed into 5fC and 5caC. The 5fC and 5caC modifications recruit the DNA base excision repair (BER) machinery. By BER activity, 5fC and 5caC are removed and replaced by new cytosine residues, free of modifications or demethylated.[10,11] It is relevant to remark that the Developmental Pluripotency-Associated Protein 3 (DPPA3) protein protects the imprinted genes and the maternal genome from the action of the TET proteins. As a result, the demethylation of the maternal genome occurs in a passive way; maternal DNA methylation is lost over successive DNA replications as a consequence of the inactivity of DNMTs proteins.[12,13] The genes regulated by imprinting are resistant to demethylation and remain with the same status (methylated or unmethylated) along development.

- *PGCs:* PGCs originate from few cells that are localized in the proximal part of the epiblast. These cells migrate in a coordinated manner along the dorsal part of the hindgut until they reach the genital ridge. During this process, DNA methylation is erased by the action of the TET proteins (TET1 and TET2) and the conversion of 5mC into 5hmC. This epigenetic reprogramming includes the genes regulated by genomic imprinting and is crucial to confer PGCs totipotency, and hence, to ensure embryo development.[14,15]
- *Spermatogenesis:* During this process, the specific epigenetic patterns of spermatozoa and the paternal allele-specific patterns of genes regulated by genomic imprinting are acquired. In humans, the establishment of methylation is considered complete at the stage of spermatogonia of the adult individual.[16] Nevertheless, additional changes can occur until the end of the pachytene spermatocyte phase.[17]

Test to Analyze Sperm DNA Methylation at Single Base-Pair Resolution

There are different approaches to analyze DNA methylation.[18] Nevertheless, the most commonly used approaches to analyze sperm DNA methylation are those that identify 5mC at single base-pair resolution. In this section, we will examine some general considerations that apply to this methodology.

General Considerations

Sperm Cell Isolation

There are several techniques to perform DNA methylation studies in ejaculated samples; however, all require the application of procedures that allow the isolation of the sperm fraction to ensure that the results specifically reflect the sperm methylation profile. A wide variety of sperm purification methods have been described.[19] Most of them are based on the selection of motile sperm and all have the purpose of selecting the sperm fraction with optimal fertilizing capacity as a prelude to the application of *in vitro* fertilization procedures. Therefore, the analysis of the sperm fraction obtained by these methods reflects the status of fertilizing sperm, not the status of the whole ejaculated sperm. An alternative is the somatic cell lysis (SCL) method,[20] which is based on the use of a mixture of detergents that results in the lysis of somatic cells but not spermatozoa. This selective analysis is based on the fact that sperm cells have a high proportion of membrane protein compared with the somatic cells present in the ejaculate. As a result, the sperm have a greater resistance to treatment with detergents. This method allows the isolation of the whole sperm fraction, including those cells with poor motility or morphology.

Sperm DNA Isolation

Sperm DNA isolation could be performed using the “classical” phenol/chloroform extraction or by commercially available kits. Spermatozoa have a high degree of chromatin compaction due to the establishment of disulfide bonds between the thiol groups of protamines. This characteristic determines the addition of one step of decondensation using a solution of 1,4-ditiotreitol (DTT) (after cell lysis). DTT breaks disulfide bonds and, therefore, reduces the compaction of chromatin and enhances the activity of treatments during the DNA isolation procedure.

Sodium Bisulfite Treatment

Under certain conditions of pH and temperature, the sodium bisulfite converts the unmethylated cytosines (C) into uracil (U) by sulfonation, desulfonation, and deamination reactions (Figure 10.2). When the modified DNA is amplified by PCR, the C residues that are methylated are amplified as C and present a guanine (G) as a complementary base. On the contrary, the nonmethylated C turned to U are amplified as thymine (T) and presented an adenine (A) as complementary base. When analyzing the

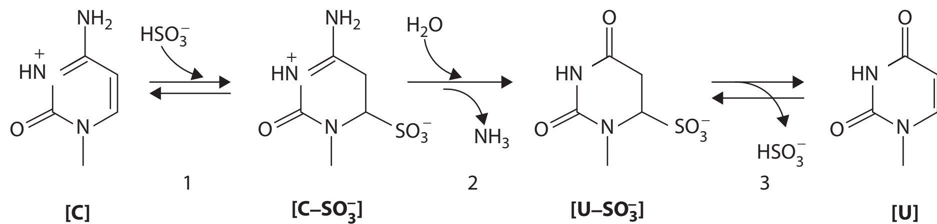

FIGURE 10.2 Unmethylated cytosine conversion by sodium bisulfite. 1, Sulfonation reaction; 2, hydrolytic deamination reaction; 3, alkaline desulfonation reaction. C, Cytosine; C-SO_3^-, sulfonated cytosine; U-SO_3^-, sulfonated uracil; U, uracil.

sequence of the PCR product, methylated/nonmethylated cytosine residues can be distinguished depending on the presence of C/G or A/T.

There are several commercial kits for the conversion of sperm DNA with sodium bisulfite. All kits are optimized to prevent the degradation of DNA, ensure the full conversion of unmethylated C to U, and protect against the conversion of methylated C.

Single-Locus Analysis

The first step for single-locus DNA methylation analysis is the amplification of the region of interest by polymerase chain reaction (PCR). Nevertheless, PCR on bisulfite-treated DNA requires some extra considerations. First, the design of the primers should take into account that they must be complementary to regions that contain at least four unmethylated cytosines susceptible to the sodium bisulfite treatment. The application of this condition ensures that primers are specifically complementary to fully converted DNA. Accordingly, in the case of inappropriate conversion, the primer would not hybridize properly avoiding nonspecific amplifications. Second, after treatment with sodium bisulfite, when a mixture of methylated and nonmethylated alleles is amplified, C or U can be found in CpG positions. In these cases, if the primers recognize regions containing CpG, the allele containing the base complementary to the amplified primer would be preferentially amplified. With this in mind, primers must be designed without CpG at the complementary region. The same applies for the presence of single nucleotide polymorphism (SNP) in the specific recognition sequence; therefore, primers should be also designed free of SNPs.

After DNA conversion and amplification, the methylation analysis of the PCR product can be performed using different strategies. The Sanger sequencing allows this kind of analysis; however, it is not a quantitative technique. As a consequence, the interpretation of methylation results is especially complicated in cases where there is a mixture of methylated and nonmethylated alleles and CpG positions are represented by double peaks corresponding to C and T. The application of cloning and sequencing, through the transformation of competent bacteria by introducing PCR products (inserts) linked to cloning vectors (plasmids), can overcome the major limitations of the Sanger sequencing. The sequencing of a single clone allows us to know the state of methylation of a single DNA molecule, avoiding the appearance of double peaks corresponding to mixtures of methylated and nonmethylated alleles. Nevertheless, it is a time-consuming and expensive procedure, usually performed in 10–15 clones per sample, which makes the identification of events that occur at low frequencies difficult.

Currently, pyrosequencing is the technique of choice for the single-locus DNA methylation analysis. Pyrosequencing is a real-time sequencing approach designed to analyze quantitatively the levels of methylation of close CpG positions.[21] After sodium bisulfite treatment each CpG could be analyzed as an SNP C/T with an allelic frequency ranging from 0% to 100%, which represents the methylation level of every CpG. Pyrosequencing provides highly reproducible and accurate information of the average of methylation of a large number of DNA molecules analyzed simultaneously. One disadvantage lies in the limited number of nucleotides that can be analyzed per reaction. Sequences longer than 80 nucleotides require the use of different primers located along the regions of interest and perform different reactions. This could be a strong limitation in sequences with large number of repetitions in which the primer design could be restricted.

Large-Scale Analysis

Large-scale analysis strategies together with gene ontology tools offers the opportunity to understand the basic mechanisms involved in the onset of complex diseases, such as male infertility. In this section, we will focus on the large-scale strategies that have been used for sperm DNA methylation studies at single-base-pair resolution: arrays and next-generation sequencing (NGS)–based techniques.

Methylation arrays allow the simultaneous quantitative methylation analysis of thousands of CpG covering different parts of the genome. This methodology is based on the ligation-dependent probe–primer pair extension. After whole-genome amplification of genomic sodium bisulfite-treated DNA, each CpG dinucleotide will be represented as CpG or TpG depending on the presence or absence of methylation. The amplification with a methylated and unmethylated specific probe–primer labeled with distinctive fluorochromes allows quantifying the signal corresponding to the specific CpG or TpG. In 2011, Illumina developed the large-scale platform Infinium HumanMethylation450 BeadChip (HM-450 BeadChip; Illumina, Inc., San Diego, California) that substantially improved, in terms of coverage and reliability, the platform 27K first developed by the company. The 450K array allows the analysis of methylation of more than 485,000 CpG per sample and covers 99% of the genes described in database, with an average of 17 CpG dinucleotides distributed along the promoter region of the gene, the first exon, the body of the gene, and the 5'UTR and 3'UTR regions.[22] Krausz et al. demonstrated the reliability of the system on sperm DNA samples.[23]

The analysis of the bisulfite-converted DNA by NGS approaches does not substantially differ from its application in nonconverted DNA. The most important differences are the necessity to analyze both strands to differentiate unmethylated CpG from SNPs (an unmethylated CpG will be read as a T and will show a G on the opposite strand) and that the alignment is performed with specific three-letter bisulfite aligner software that uses in silico bisulfite-converted reference genome. Molaro et al. satisfactorily applied this technology to explore the human and chimp sperm DNA methylation.[24]

Characteristics of the Sperm Methylome

New technologies addressed to analyze whole-genome DNA methylation and histone modifications are being applied in defining the epigenome of different cell types,[25] including human spermatozoa.[23,24] As we state earlier, the sperm methylome is the result of a series of reprogramming events that occur during spermatogenesis and early embryogenesis. Taking into account the crucial functions in which DNA methylation is involved, a correct sperm epigenome is essential for sperm function and male fertility.

In recent years, the application of large-scale strategies has allowed the elucidation of some insights into DNA methylation features of human spermatozoa. Data show that the sperm methylome is conserved among samples with most of the CpGs in a hypomethylated or hypermethylated state but clearly polarized toward hypomethylation.[23,24] These results suggest that the progression of germ cells throughout spermatogenesis requires a strict control of the CpGs methylation levels.

Focusing on conserved CpGs, some authors have found that most of the hypomethylated-conserved sites were associated to promoter regions. It has been reported that in sperm the promoters of crucial genes involved in early embryonic development are hypomethylated, histone-retained, and enriched in trimethylation of histone H3 on lysine 4 (H3K4me3) marks.[23,26] This feature has been associated as a mechanism ensuring their rapid activation in the early embryo, suggesting that promoters are not reprogrammed after fertilization. Interestingly, this correlation with embryonic developmental genes appears to be missing when all conserved hypomethylated regions are analyzed, including histone-depleted regions.[23]

Origin and Consequences of Sperm DNA Methylation Defects

The knowledge of the sperm methylome is the first step to identify possible variations causing male infertility. As we state before, changes in the methylation pattern could cause changes in gene expression, which initially would modify the transcriptome, leading to fertility problems. Actually, the influence of

sperm DNA methylation on the reproductive capacity of couples has been postulated as an explanation for male infertility.[27] The pattern of methylation in mature sperm reflects changes in the pattern of gene expression that occurs during spermatogenesis. Because DNA methylation controls the transcriptional activity of genes and is also involved in establishing higher-order chromatin structure and genomic stability, the fidelity of this process determines whether the progression of meiosis occurs properly, culminating in the production of functional spermatozoa. Thus, abnormalities in erasure or establishment of DNA methylation may affect sperm production, both quantitatively and qualitatively, and could explain some cases of male infertility.

Origin of Sperm Methylation Defects

A number of studies have demonstrated that sperm DNA methylation is sensitive to lifestyle and the individual genetic background.[28] Recent studies showed that exercise training,[29] diet,[30–35] or the exposure to endocrine disruptors[36,37] affects the sperm methylome. Variations can also arise because of an intrinsic age-related factor; some authors have demonstrated a significant increase in hypermethylated spermatozoa with age.[38–40] Moreover, the implication of intrinsic genetic-related factors through the mutations or polymorphisms of genes related to the metabolic pathway for obtaining methyl groups, or in the mechanisms involved in DNA methylation erasure, establishment, and maintenance has been also reported.[33,35,41]

All the factors exposed in the preceding paragraph, individually or in concert, are potential causes of sperm DNA methylation variations that could affect the fertility of the patients or be a cause of disease in the offspring.

Consequences of Sperm DNA Methylation Defects on Fertility

Several pieces of data have suggested a relationship between aberrant sperm DNA methylation, altered gene expression, and male infertility. In particular, there have been reports of alterations of imprinted genes,[41–51] spermatogenesis critical genes such as *DAZL*[52] (Deleted In Azoospermia Like) and *CREM* (CAMP Responsive Element Modulator),[53] ALU regions of repetitive DNA,[149,54] and even genes without a direct connection to spermatogenesis.[54–59] All these papers suggest that sperm DNA methylation patterns differ significantly between infertile and fertile, highlighting the possibility that aberrant sperm DNA methylation may lead to decreased fertility.

Concerning the role that abnormal sperm methylation plays after fertilization, several data suggest the influence of the sperm epigenome on embryogenesis. As we stated previously, it has been reported that the promoters of crucial genes involved in early embryonic development are hypomethylated in sperm.[23,26] Several animal experiments confirm that the offspring of epigenetic profile and health status are influenced by paternal preconceptional insults, such as exposure to endocrine disruptors or toxins[60]; ionizing radiation[61]; and nutritional status.[62–64] This phenomenon has been also observed in human epidemiological studies.[65–67] Accordingly, altered epigenetic profiles in the sperm could have profound effects on embryonic development and pregnancy outcome in patients submitted to assisted reproductive technology (ART). In this context, only a few studies have compared the relationship between sperm DNA methylation and ART outcome. Some authors have demonstrated that abnormal sperm DNA hypomethylation is associated with poor pregnancy rate.[68] It has been shown that sperm samples leading to abortions showed significantly lower ALU methylation levels than those leading to the birth of a baby.[49] Other data come from the study of the methylation status of imprinted genes; there are studies linking poor embryo quality with sperm methylation alterations of KvDMR (Kv-differentially methylated region) and SNRPN-ICR[51] (Small Nuclear Ribonucleoprotein Polypeptide N - Imprinting Control Region); this association has been also observed between lower fertilization rate and altered methylation pattern for *IGF2* (Insulin Like Growth Factor 2) and *H19*,[46] although a recent publication did not find any relationship.[69]

If we considered that PGCs are extensively reprogrammed during development (see the section "Epigenetics: DNA Methylation"), it could be argued that sperm DNA alteration will be reprogrammed and thus will not affect future generations. However, it is well known that during epigenetic reprogramming events, DNA methylation is retained at regions of repetitive DNA to keep them inactivated. Moreover, a comparison of mouse oocyte and sperm methylomes has identified a significant amount of differentially methylated CpG islands (in nonimprinted regions) that are partially resistant to the

global DNA demethylation that occurs during preimplantation development.[70] Recently, the retention of methylation during PGCs and preimplantation embryo epigenetic reprogramming in nonrepetitive regions of the genome has been described in humans. Some of these loci are associated with genes that have been related with metabolic and neurological disorders, being candidates for transgenerational epigenetic inheritance.[15] The intrinsic and/or extrinsic or environmental factors that may influence an individual's epigenetics at a somatic level can also influence their germline cells, affecting regions of the genome that are not reprogrammed after fertilization. Therefore, epigenetic variations present in the parents could be transmitted to offspring.[71]

Final Remarks

Future genome-wide methylation studies, in larger and well-defined cohorts of infertile patients, are mandatory to identify the molecular mechanisms evoking variations in the sperm methylome. These studies would provide better insights into the association between sperm DNA methylation patterns and male infertility, and might allow the identification of epigenetic fertility biomarkers.

Although the relationship between aberrant methylation and male infertility seems to become relevant, their relationship with the presence of epigenetic abnormalities in the offspring is dependent on long-term, large-scale, and complex population studies in children conceived by ART.

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11

The Use of Sperm Ultra-Morphology Assessment in Assisted Reproduction

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Introduction

Semen examination is one of the key steps in the exploration of infertile couples. The normal ejaculate contains spermatozoa with significant changes in the size and shape of the head, the acrosome, and the intermediate part of the flagellum. This diversity is explained by the fact that the spermatozoon is the ultimate result of a highly complex process of differentiation that occurs during spermatogenesis and is completed by a morphological and functional maturation during the epididymal transit. These processes can be the target of endogenous or exogenous toxic factors that can induce excessive production of morphologically abnormal sperm responsible for teratozoospermia. An abnormal sperm morphology can compromise the fertilizing ability of human spermatozoa. Indeed, morphological abnormalities appear to be higher in infertile males compared with fertile males and sperm morphology is of major interest in male infertility diagnosis.[1] However, the morphological profile of the semen sample seems to be the most constant semen parameter in the same male.[2–4]

Sperm morphology has been recognized as the best predictor of natural fertility or after in vitro fertilization (IVF) or intrauterine insemination.[5,6] However, most published data failed to clearly demonstrate a relationship between sperm morphology and intracytoplasmic sperm injection (ICSI) outcome.[7–9] Several explanations have been proposed: (1) a normal sperm morphology is required to penetrate the oocyte barriers, which are bypassed by ICSI, (2) spermatozoa with obvious morphological abnormalities are excluded from ICSI, (3) spermocytogram performed on randomly selected spermatozoa from an entire ejaculate is not an accurate assessment of the quality of each injected spermatozoon, and (4) ultrastructural sperm anomalies that are the only ones associated with ICSI outcome cannot be detected using either the ×100 or the ×200–400 magnifications.[10]

Sperm morphology is only one qualitative parameter of spermatogenesis, and the conventional sperm morphological analysis is a descriptive exploration of sperm shape from a representative sample of spermatozoa from one ejaculate. Furthermore, sperm morphology analysis depends on imaging techniques and criteria used to define a morphologically normal sperm. To improve the diagnosis of male infertility, several methods have been successively proposed to objectively assess sperm morphology.

Light and Electron Microscopy

The morphological profile of a semen sample is the most constant parameter in the same man[2–4] and can be explored by several imaging techniques, each one with its own specific criteria to define a normal spermatozoon. Light microscopy is the most common imaging system used in routine in reproductive biology laboratories and allows after staining of fixed sperm preparations the classification of morphologically normal or abnormal spermatozoa. Three main classifications are currently used. The first is the David classification,[2] mostly used in French reproductive biology laboratories, which accurately describes 15 types of anomalies (seven for the head, three for the intermediate piece, five for the flagellum) and for which the rate of typical forms (normal sperm) should be greater than 50%.

No data are, however, given concerning sperm head vacuoles despite their demonstrated presence since several years[11]; The second, the Krüger classification, identifies the same abnormalities as described by the David. However, it takes into account each anomaly individually, while the David classification considers all anomalies for the same spermatozoon. The definition of sperm normalcy criteria is stricter than for the David classification.[12] Thus, all the spermatozoa considered with a borderline morphology within the David classification are classified as atypical for the Krüger classification. Therefore, this classification defines teratozoospermia when the semen sample contains less than 14% of typical sperm forms. Again, no information is given about the presence of vacuoles in sperm heads. The third classification, the World Health Organization (WHO) classification, is based on the previous classification but it sets the threshold percentage of typical spermatozoa to 30%.[13] More recently, sperm head morphology with vacuoles was evaluated after Papanicolaou staining and observation under a light microscope (×400) in sperm samples from 980 males. Paradoxically, the proportion of sperm head vacuoles increased with the ratio of normal sperm morphology. This observation led to the conclusion that morphologically normal sperm possess vacuoles in their heads.[14]

Nevertheless, given the final magnification obtained by light microscopy (×1000), only a description of the sperm surface is carried out. No clear description of the organelles and the sperm nucleus can be obtained, parameters that play a major role in male fertility.

The evaluation of the integrity of the different structures of the male gamete by transmission electron microscopy (TEM) or scanning electron microscopy (SEM) is important in sperm ultramorphology assessment. TEM allows viewing two-dimensional (2D) or detailed sections of spermatozoa at a magnification ×35,000, such as the acrosome, postacrosome, or the integrity of the individual components of the flagellum. However, SEM gives a better evaluation of the three-dimensional (3D) structure of the spermatozoon. The scanning morphogram is achieved by giving a particular attention to the head and skeleton flagellum.[15] To render this ultrastructural sperm analysis suitable for a clinical application to differentiate between fertile and infertile men, Bartoov et al.[15] established an ultrastructural quantitative index called quantitative ultramorphological index (QUM = [% of normal nuclei] × 0.04 − [% of abnormal acrosomes × 0.032] − [% of abnormal dense fibers × 0.044] − 0.07). This index can be considered a positive predictor of fertility in 75% of couples. By combining QUM with conventional semen parameters, the positive predictive value rises by 80%. This expensive technology is not available in routine semen analysis laboratories. However, the QUM may be useful for patients after intrauterine insemination or IVF failure despite normal conventional sperm parameters.[15]

Motile Sperm Organelle Morphology Examination

All the aforementioned imaging techniques that are used for the assessment of sperm morphology require fixed nonviable spermatozoa and do not allow a real-time study of living and motile spermatozoa.

In 2002, Bartoov et al.[10] introduced a light imaging system called motile sperm organelle morphology examination (MSOME) that observes spermatozoa in real time and at a high magnification. They used an inverted microscope equipped with Nomarski differential interferential contrast microscopy and a ×100 immersion objective. This microscope is also equipped with a 3D camera and a color monitor. The final magnification that takes into account the microscope lens and the zoom of the video monitor is close to ×6600. It allows the analysis in real time of isolated motile spermatozoa placed into a petri dish with glass bottom at high magnification (up to ×6600). The different parts of the spermatozoon such as the acrosome, the postacrosome, the neck, the middle piece, the flagellum, and the nucleus are described. A normal spermatozoon has an oval head, which is smooth and symmetrical; its size varies between 4.75 ± 0.28 μm in length and 3.28 ± 0.2 μm in width. These criteria were defined arbitrarily using the previous examination of TEM studies. Chromatin is considered abnormal if more than one vacuole occupies more than 4% of the total sperm head surface. A normal head should have a normal shape and a normal content.[10,15,16] MSOME is able to identify not only solely conventional morphological sperm alterations with a definition close to SEM but also more specifically sperm head vacuoles, considered by Bartoov et al. (2001)[16] to be nuclear defects. The specific sperm morphological abnormalities found in MSOME are summarized in Table 11.1.

TABLE 11.1

Spermatozoa Morphological Specific Abnormalities Detected after High-Magnification Motile Sperm Morphology Examination (MSOME)

	Acrosome	**Postacrosome**	**Nucleus**		**Neck**	**Flagellum**	**Mitochondria**
			Form	**Chromatin**			
Specific morphological abnormalities	Absent, Partial Vacuole	Absent Vacuole	Microcephalia Macrocephalia Thinned (width <2.9 µm) Large (width ≥3.7 µm) Short (length <4.2 µm) Irregular	Relative vacuolar area > 4% of head area	Abnormal axis Irregular Cytoplasmic rest	Absent Angulated Irregular Multiple Short	Absent Partial Disorganized

It is necessary to define MSOME parameter normal values in sperm evaluation as part of routine male infertility diagnosis. As mentioned earlier, MSOME parameters proposed by Bartoov et al.[10] were based on TEM and SEM observations and were not confirmed using MSOME observation. No consensus has been established for a specific, objective, and reproducible procedure to obtain a classification of abnormal MSOME spermatozoa. However, MSOME enables better detection of sperm head vacuoles considering their size (small or large), their number, and position (anterior, median, or posterior).[17] The procedures used are generally subjective, performed by visual observation, with no specific measurements of sperm head vacuoles. An objective and precise measure of the vacuole was performed in our laboratory and conducted to define the relative vacuolar area (RVA), expressed as the ratio between the global surface of the vacuole(s) and the area of the sperm head (%). The RVA was considered normal when it occupied less than 6% of head area[17] or less than 6.5%.[18] The same value of RVA was recently reported by another team using the same objective procedure to assess the RVA in a control population consisting of 50 healthy fertile men aged 21 to 41 years with proven spontaneous fertility.[19] The classification of normal MSOME spermatozoa did not vary from these published data.[20] The normal value of RVA of less than 6.5%[17–19] is close to the value of 4% proposed initially by Bartoov et al..[10]

Several classifications have been proposed to define abnormal MSOME parameters. Most of the laboratories used a rapid and subjective visual screening of sperm head vacuoles based on the criterion proposed by Bartoov et al.[10] with a high risk of variability between observers. Four grades of sperm head abnormalities have been proposed considering the absence (Grade I) or the presence (Grade IV) of large vacuoles associated with abnormal head shapes or other abnormalities.[21,22] Cassuto et al.[23] used MSOME to analyze more than 15,000 spermatozoa from 100 randomly selected patients. They proposed an analysis of sperm head shape to obtain a score per spermatozoon that takes into account the presence of vacuoles and abnormal head shape or head base with "class 1" for high-quality spermatozoa (score 4–6) and "class 3" for low-quality spermatozoa (score 0). Mauri et al.[24] defined five grades of spermatozoa in a population of 30 infertile males with semen parameter impairment, with "Grade I" for normal spermatozoa as proposed by Bartoov et al.,[16] and "Grade IV" and "Grade V" spermatozoa for spermatozoa presenting large vacuoles occupying 5%–50% and more than 50% of sperm head area, respectively. Using objective measurement of sperm head vacuoles in a population of 331 infertile and 109 fertile males, respectively,[17] and a population of 50 fertile males and 51 infertile males with idiopathic infertility,[19] "type 1" spermatozoa were considered normal with an RVA less than 6.5% and "type 4" spermatozoa were considered abnormal with a large RVA occupying more than 12.4% of the sperm head area.

Factors That Might Affect the Occurrence of Sperm Nuclear Vacuoles

The mean percentages of morphologically normal spermatozoa and spermatozoa with large nuclear vacuoles did not vary between the two different MSOME analyses performed on two semen samples from 240 men from an unselected group of couples undergoing infertility investigation and treatment.[25,26] MSOME seems to be a stable method for at least these two specific sperm forms within the context of semen exploration of infertile males. However, in a population of 56 male patients with infertility who underwent multiple semen analyses and high-magnification observation of the sperm head, the proportion of spermatozoa with large nuclear vacuoles varies significantly even if moderately between the two ejaculates.

The impact of temperature (incubation, high or low temperature) on the occurrence of sperm nuclear vacuoles is controversial. It appears that initially after 2 hours of incubation at 37°C, a significant increase in the frequency of sperm-vacuolated nuclei occurred, recommending preferential incubation of spermatozoa at 21°C.[27] However, more recently, it has been proposed that the temperature itself during incubation did not influence the degree of sperm nuclear vacuolization of motile sperm; rather it is the mode of sperm preparation that might induce sperm nuclear vacuolization at 37°C. Therefore, sperm nuclear vacuolization is unaffected by temperature in motile sperm after preparation and isolation by swim-up.[28] We can conclude that the variation in temperature or environmental conditions in the laboratory has no impact on the formation or disappearance of vacuoles.[29] Furthermore, cryopreservation induced sperm nuclear vacuolization by increasing the proportion of Grades III and IV spermatozoa presenting sperm with large nuclear vacuoles. Cryopreservation seems to alter the organelle morphology of motile human spermatozoa.[30] In a more recent study, there was no evidence for any difference in any vacuolar criteria such as relative vacuole area and vacuole location, leading to the conclusion that freezing–thawing procedures have no effect on human sperm vacuoles.[31]

The rate of spermatozoa carrying small or large vacuoles occupying less or more than 4% of sperm head area increases with patient's age.[32,33] The proportion of spermatozoa with large nuclear vacuoles slightly decreased among the 18 patients who underwent varicocele repair.[34]

Characteristics and Origin of Sperm Head Vacuoles

Sperm head vacuoles vary in size, number, and location. They may be small or large, single or multiple, shallow or deep lying. Furthermore, sperm head vacuoles are relatively common in spermatozoa from fertile males with normal semen parameters[17,19,35] or in infertile males with normal and abnormal semen parameters.[17,19,22,36,38] The prevalence of vacuoles varies from 34% to close to 99% depending on the reported studies (for review, see Perdrix et al.[37] and De Vos et al.[38]).

Small vacuoles are relatively frequent in normally shaped spermatozoa.[35,36] Large nuclear vacuoles occupying more than 13% or 50% of the sperm head are more prevalent in semen samples of infertile men presenting preferentially a high level of polymorphic teratozoospermia.[17,18] Small nuclear vacuoles with an RVA close to those observed in a reference population of fertile males were detected in spermatozoa of two patients with globozoospermia.[39] In fertile and infertile males, small or large vacuoles are mainly located in the anterior or median part of the sperm heads[18,36,40,41] and more exceptionally in the posterior part.[36] The number of vacuoles per spermatozoon varies between 1.5 and 2.1.[17,19,36]

Some studies suggested that these vacuoles are physiological constituents of spermatozoa without impact on their functionality.[36] Other studies defended the hypothesis of their acrosome origin by assessing vacuole parameters after induction of the acrosome reaction.[42,43] Furthermore, MSOME analysis in the case of complete globozoospermia demonstrated the presence of sperm head vacuoles without any acrosomal or Golgi residues.[39] Recently, however, the continuous observation of the same living sperm before and after acrosome reaction induction did not change either the proportion of spermatozoa with vacuoles or the disappearance of preexisting vacuoles.[29] It also appears that the degree of sperm nuclear vacuole content was significantly lower in acrosome-reacted spermatozoa and motile spermatozoa compared with immotile sperm that were not acrosome reacted.[44]

The different studies performed with confocal microscopy, TEM analysis, or atomic force microscopy on selected or unselected spermatozoa with large or small vacuoles confirmed that these vacuoles are large or small pocket-like, 4,6-diaminidino-2-phenylindole (DAPI) negative, and thus DNA-free concavities and exclusively of nuclear origin.[18,40,41,45–47,] The vacuoles are covered by acrosomal and plasmic membranes of the sperm head. These nuclear lacunae correspond to localized uncondensed chromatin areas[18,41,45,46,48] due to protamine deficiency.[41,49] Nuclear vacuoles may be generated during the highly complex process of chromatin condensation that occurs during spermiogenesis. The subsequent abnormal and imperfect replacement of histones by transition proteins and protamines in localized areas may lead to an incorrectly condensed chromatin and the genesis of nuclear vacuoles. Such nuclear vacuoles have been previously reported during spermiogenesis in elongated and late spermatids in normal and pathological context.[36,50] The abnormal sperm head condensation during spermiogenesis may interfere with the normal achievement of sperm nuclei condensation during epididymal transit and may expose them to DNA damages.

Sperm Vacuoles and Sperm DNA Damage

The presence of large sperm head vacuoles has been suspected to be related to sperm DNA damage. The characterization of the specific DNA defects associated with large sperm head vacuoles has been assessed on isolated spermatozoa and more largely on spermatozoa from the whole semen samples. Most of these studies explored large sperm head vacuoles with various criteria to define the RVA of large vacuoles: vacuole area can occupy more than 4% of head area,[42,49,51] 13%,[18] 25%,[48] 50%,[52] or vacuole area >1.5 µm, and is visible at ×400 magnification,[35] or not precisely described.[45] Only one study investigated small sperm head vacuoles. The patients included in the different published studies were fertile,[35] often infertile,[42,52] or with semen parameter alterations.[17,18,35,45,48,49]

Semen samples containing high level of spermatozoa with large nuclear vacuoles or isolated spermatozoa with large nuclear vacuoles presented a high level of chromatin immaturity.[18,45,46,48,52,53] Furthermore, small nuclear vacuoles were also considered pocket-like nuclear concavities related to failure of chromatin condensation.[40,41] This abnormal chromatin condensation was also confirmed by the modification of chromosome architecture and positioning in spermatozoa with large vacuoles.[37] However, the presence of sperm head vacuoles in 873 males enrolled in assisted reproductive procedure was not associated with abnormal DNA condensation or DNA fragmentation.[54]

Therefore, it appears that sperm with large vacuoles or semen samples with a high rate of spermatozoa carrying large nuclear vacuoles presented an increase of DNA fragmentation compared with normal spermatozoa or normal semen samples,[25,32,45,51,52,55] but these data were not confirmed in other publications.[18,35,48,53,55] Hence, sperm nuclear vacuoles might be associated with DNA fragmentation when spermatozoa came from semen samples with a high level of DNA damage that might be the consequence of an abnormal microenvironment that impairs, not solely, the normal condensation of the nucleus during spermiogenesis and consequently exposes the sperm DNA to damage during the epididymal transit.[40,41]

Sperm aneuploidy explored by fluorescent in situ hybridization (FISH) or more exceptionally by using human sperm karyotypes after sperm injection into mouse oocyte reported an increase of aneuploid sperm content in association with the presence of large vacuoles, even if the rate did not reach the significance.[17,35,45,48,] De Almeida et al. (2010) did not confirm this relationship. Furthermore, we observed a significant increase in sperm aneuploidy when compared with the native semen sample.[18] However, no relationship was established between sperm nuclear large vacuoles and the chromosome content in the case of large sperm head syndrome[56] or in the case of patients carrying reciprocal or Robertsonian translocation.[57]

From MSOME to Intracytoplasmic Morphologically Sperm Injection

The MSOME method was used thereafter to select morphologically normal spermatozoa without vacuoles before ICSI, giving rise to intracytoplasmic morphologically selected sperm injection (IMSI).

The first publication reported an increase in pregnancy rate using IMSI compared with that using ICSI; this was also reported by Bartoov et al.[10,16,58] The impact of nuclear sperm normal morphology on ICSI success was highlighted.[59] The role of sperm vacuoles observed with MSOME in assisted reproductive technology (ART) was questioned.[60] However, more than 10 years after these first publications, the real benefit of the use of IMSI rather than ICSI to improve the ART success rate has not been clearly demonstrated because different studies lead to contradictory conclusions.

Most of the studies that evaluated the benefits of IMSI instead of ICSI or other ART procedures were retrospective and nonrandomized studies. IMSI cycles were generally paired with previous ICSI cycles for the same infertile couples or ICSI cycles performed in the same period in other infertile couples (Table 11.2). These nonrandomized and retrospective trials showed that fertilization rate was not improved by IMSI except for five studies.[23,61–63] In one study comparing the advantage of IMSI rather than ICSI in poor responder patients, fertilization rate, the proportion of cycles with embryo transfer, and the number of transferred embryos were significantly lower in IMSI compared with ICSI; it appears that poor responder patients do not benefit from IMSI.[64] Considering that sperm head vacuoles are associated with abnormal chromatin condensation, it has been proposed that injection of normal spermatozoa without large head vacuoles enhanced embryo quality and morphology not only at an early stage[60,61,65,66] but also at the blastocyst stage.[21,23,65,67] In these retrospective studies, IMSI improved the clinical pregnancy rate[58,60–62,66,68,69] and reduced the miscarriage rate.[58,60,61,66] In addition, IMSI pregnancies were less likely to involve a fetal birth defect compared with ICSI, even if not significantly so. IMSI did not involve an increase in malformation rate and may have reduced anomaly incidence.[69] Setti et al. (2014)[70] performed a meta-analysis on IMSI outcomes and showed that IMSI increases the odds of implantation by 50% and pregnancy by 60% in couples with male factor in fertility. Furthermore, IMSI results in a threefold increase in implantation rate, a twofold increase in pregnancy rate, and a 70% decrease in miscarriage rate in couples with ICSI failure compared with couples having a success using ICSI. They recommend promoting IMSI in couples with male factor and also with ICSI failure. However, they also concluded that randomized studies are needed to confirm the IMSI benefits under such conditions.

The data obtained from prospective randomized or non-randomized trials also lead to contradictory conclusions (Table 11.3). However, prospective studies and more specifically randomized trials provide the most robust evidence to evaluate the real benefit of IMSI compared with ICSI. Only one prospective study has demonstrated the benefit of IMSI to increase the fertilization rate.[63] Two studies showed a lower rate of fertilization with IMSI compared with ICSI.[72,73] Only two studies reported an advantage of IMSI to improve the number and quality of early cleaved embryos.[51,63] Five studies demonstrated that IMSI enhances the clinical pregnancy rate.[24,74–77] The miscarriage rates did not vary significantly between IMSI and ICSI (Table 11.3). Delaroche et al.[63] demonstrated in a prospective comparative study evaluating IMSI after IVF or ICSI failures that IMSI gave better embryo quality and more blastocysts that allow more embryo transfers at the blastocyst stage. They recommend the use of IMSI after ICSI or IVF repeated failures. However, Leandri et al.[72] indicated that ICSI has no advantage in the first ART attempts for male infertility factor. In addition, Teixeira et al.,[78] in the Cochrane database, enhanced the fact that there is no evidence of effect of IMSI on live birth or miscarriage and the evidence that IMSI improves clinical pregnancy is very poor.

In conclusion, more than 10 years after the first description of MSOME and IMSI as potential new tools in ART, the presence of large vacuoles in the sperm head has been suspected, but not demonstrated, to have deleterious effects on the outcomes of ART. MSOME appeared initially as a helpful tool for fine and precise sperm morphology assessment and its application might have enhanced the ART success rates. However, to the best of our knowledge, MSOME is not routinely proposed in most ART laboratories because of the absence of a well-standardized MSOME analysis.

IMSI has been proposed initially in infertile couples after repeated ART procedure failure. Thereafter, it has been suggested that IMSI might replace ICSI. However, no study has demonstrated that IMSI gave better ART outcomes compared with ICSI. Therefore, it has been postulated that IMSI should be proposed in particular indications such as severe teratozoospermia or severe semen parameter impairment. However, none of these indications has been validated in randomized clinical trials. To potentially validate such indications, randomized prospective and multicenter clinical trials are necessary to be able to include a high-level number of patients. The benefit of IMSI over ICSI remains controversial.

TABLE 11.2

Retrospective Studies Comparing Intracytoplasmic Morphologically Sperm Injection (IMSI) and Intracytoplasmic Sperm Injection (ICSI) According to Different ART Parameters

			IMSI Parameters				
				Embryo Quality			
Studies	**Studied Population**	**Methodology**	**Fertilization**	**% of Good Quality Early Cleaved Embryos**	**% of Good Quality Blastocysts**	**Clinical Pregnancy Rate**	**Miscarriage Rate**
Bartoov et al. (2001)[16]	24 couples, male infertility Woman < 37 years At least three retrieved oocytes At least 5 IVF-ICSI failures	Comparison with previous ICSI attempt	NS			IMSI = 58% (versus 3% expected with ICSI)	
Bartoov et al. (2003)[58]	62 couples, altered semen analysis Woman < 37 years At least three retrieved oocytes At least two ICSI failures	Comparison to 50 control couples, paired according the number of previous ICSI failures	NS	NS		IMSI = 66% ICSI = 30% $p < 0.01$	IMSI = 9% ICSI = 33% $p < 0.01$
Hazout et al. (2005)[68]	125 couples Woman < 38 years 37 patients with normal semen analysis 88 patients with altered semen analysis At least two ICSI failures	Comparison with previous ICSI attempt	NS	NS		IMSI = 40.8% ICSI = 6.4% $p < 0.001$	
Berkovitz et al. (2006a)[60]	28 couples Woman < 40 years At least three retrieved oocytes	Comparison of 28 IMSI with large vacuoles spermatozoa injection (a) to 28 paired IMSI with normal spermatozoa injection (b)	NS	NS		IMSI (a) = 18%(°) IMSI (b) = 50%(°) $p = 0.01$	IMSI (a) = 80% IMSI (b) = 7% $p = 0.01$

(*Continued*)

TABLE 11.2

Retrospective Studies Comparing Intracytoplasmic Morphologically Sperm Injection (IMSI) and Intracytoplasmic Sperm Injection (ICSI) According to Different ART Parameters (*Continued*)

			IMSI Parameters				
				Embryo Quality			
Studies	**Studied Population**	**Methodology**	**Fertilization**	**% of Good Quality Early Cleaved Embryos**	**% of Good Quality Blastocysts**	**Clinical Pregnancy Rate**	**Miscarriage Rate**
Berkovitz et al. (2006b)[64]	80 couples At least two ICSI failures	Comparison to 80 control couples, paired according the number of previous ICSI failures	NS	IMSI = 38.7% ICSI = 25.7% $p < 0.05$		IMSI = 60%(°°) ICSI = 25%(°°) $p < 0.05$	IMSI = 14% ICSI = 40% $p < 0.05$
		Comparison of 70 IMSI with first choice spermatozoa injection (a) to 70 paired IMSI with second choice spermatozoa injection (b)	IMSI (a) = 74.1% IMSI (b) = 62.3% $p < 0.05$	IMSI (a) = 26.7% IMSI (b) = 16.2% $p < 0.05$		IMSI (a) = 58.6%(°) IMSI (b) = 25.7%(°) $p < 0.05$	IMSI (a) = 9.8% IMSI (b) = 33.3% $p < 0.05$
Vanderzwalmen et al. (2008)[21]	25 couples, male infertility Woman < 40 years At least eight retrieved oocytes	Comparison of the results obtained according to the type of spermatozoon injected: Grade I (a), Grade II (b), Grade III (c) or Grade IV (d)	NS	NS	IMSI (a) = 37.5% IMSI (b) = 37.1% IMSI (c) = 1.7 IMSI (d) = 0% $p < 0.001$		
Cassuto et al. (2009)[23]	27 couples, male infertility Woman < 36 years At most, two IVF-ICSI failures Semen alterations	Comparison of the results obtained according to the type of spermatozoon injected: Class 1 (a), Class 2 (b), or Class 3 (c)	IMSI (a) = 84% IMSI (b) = 73% IMSI (c) = 61% $p < 0.04$		IMSI (a) = 15% IMSI (b) = 9% IMSI (c) = 0% $p < 0.03$		

Nadalini et al. (2009)[65]	20 couples Woman < 38 years Male infertility factor and/or more than 1 ICSI failure At least 5 mature retrieved oocytes	Comparison with 37 ICSI paired couples	IMSI = 91.67% ICSI = 78.33% $p = .043$			IMSI = 40.00%(°°) ICSI = 16.21%(°°) $p = .05$	NS
Oliveira et al. (2011)[85]	100 couples Woman < 39 years At least two ICSI failures, with a satisfying embryo quality	Comparison with 100 ICSI paired couples	NS	NS		NS	NS
Setti et al. (2012a)[65]	332 couples >4 and <30 oocytes retrieved Exclusion of semen samples presenting <1 million/mL of spermatozoa or <20% of motile spermatozoa	Comparison to 332 ICSI paired couples	NS	<0.001	0.001		
Delaroche et al. (2013)[63]	75 couples At least two IVF or ICSI failures	Comparison with previous IVF or ICSI attempt	IMSI =7 2.2% Control cycle = 63.3% $p = 0.02$	IMSI = 89.8% Control cycle = 79.8% $p = 0.009$	IMSI = 1.5(*) Control cycle = 1(*) $p = 0.03$	NS	
Klement et al. (2013)[69]	1302 couples Male infertility factor First IVF treatment	Comparison of 269 IMSI with 1033 ICSI				NS	
	449 couples Male infertility factor Second IVF treatment (post-ICSI failure)	Comparison of 127 IMSI with 322 ICSI				IMSI = 56% ICSI = 38% $p = .002$	
Shalom-Paz et al. (2014)[66]	42 couples Women infertility Least three previous failure cycle IVF-ICSI Poor sperm quality	Comparison of 80 cycles, 53 IMSI with 27 IVF-ICSI	IMSI = 50.0% IVF-ICSI = 44.4% NS	IMSI = 53% IVF-ICSI = 40% $p = 0.076$		IMSI = 41.3% IVF-ICSI = 10.5% $p = 0.02$	IMSI = 34.7% IVF-ICSI = 0% $p = 0.003$

(Continued)

TABLE 11.2

Retrospective Studies Comparing Intracytoplasmic Morphologically Sperm Injection (IMSI) and Intracytoplasmic Sperm Injection (ICSI) According to Different ART Parameters (*Continued*)

			IMSI Parameters				
				Embryo Quality			
Studies	**Studied Population**	**Methodology**	**Fertilization**	**% of Good Quality Early Cleaved Embryos**	**% of Good Quality Blastocysts**	**Clinical Pregnancy Rate**	**Miscarriage Rate**
Setti et al. (2015)[67]	414 matched cycles Group poor responder (< or = 4 oocytes retrieved) Group normo-responder (>4 oocytes retrieved) Patients IMSI were matched with patients who underwent ICSI in the same period	Comparison of 207 IMSI with 207 ICSI	NR-group IMSI = 72.3% ICSI = 75.9% $P = 0.107$ PR-group IMSI = 53.9% ICSI = 79.8% $p < 0.001$	NR-group IMSI = 41.1% ICSI = 45.8% $p = 0.122$ PR-group IMSI = 57.4% ICSI = 48.7% $p = 0.314$		NR-group IMSI = 34.1% ICSI = 39.4% $p = 0.355$ PR-group IMSI = 22.2% ICSI = 11.8% $p = 0.314$	NR-group IMSI = 8.7% ICSI = 7.1% $p > 0.999$ PR-group IMSI = 33,3% ICSI = 0.0% $p = 0.433$
Luna et al. (2015)[67]	31 couples Preimplantation genetic diagnosis Male and female infertility	Comparison of 11 IMSI with 20 ICSI	IMS I = 42% ICSI = 93% NS	IMSI = 39% ICSI = 68 $p < 0.001$	IMSI = 14% ICSI = 18% $p < 0.001$	IMSI = 50% ICSI = 43% NS	IMSI = 0% ICSI = 33% NS
Gatimel et al. (2016)[71]	216 couples Post-ICSI failure (two ICSI attempts)	Comparison of 216 IMSI with 532 ICSI				IMSI = 23% ICSI = 21% NS	

Abbreviations: ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection; IMSI, intracytoplasmic morphologically selected sperm injection; IVF, in vitro fertilization; NR, normo-responder; NS, not significant; PR, poor responder.

(*), number of blastocysts obtained; (°), clinical pregnancy rates per cycle; (°°), clinical pregnancy rates per embryo transfer.

TABLE 11.3

Prospective Studies Comparing Intracytoplasmic Morphologically Sperm Injection (IMSI) and Intracytoplasmic Sperm Injection (ICSI) According to Different ART Parameters

		Comparison between IMSI and ICSI											
		Fertilization Rate (%)			Embryo Quality (% of Good Quality Early Cleaved Embryos)			Clinical Pregnancy Rate (*)/Cycle (%)			Miscarriage Rate (%)		
Studies	**Studied Population**	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***
Antinori et al. (2008)[74]	446 couples (219 ICSI, 227 IMSI) OAT Primary infertility <3 years Woman <35 years							26.5	39.2	0.04	24.1	16.9	NS
	After randomization, complementary study, according to ART history: Subgroup (A): no previous failure of ICSI Subgroup (B): one previous failure of ICSI Subgroup (C): two or more previous failures of ICSI							(C): 12.9	(C): 29.9	(C): 0.017			
Mauri et al. (2010)[24]	30 couples At least two semen parameters altered or implantation failure in ICSI Randomized oocytes	70.9	70.4	NS	(D2): 57.8	(D2): 52.2	NS						
Figueira et al. (2011)[87]	120 couples with IVF associated with preimplantation genetic screening for advanced maternal age (60 ICSI, 60 IMSI) Exclusion: less than six retrieved oocytes	84.3	76.7	NS				47.1	53.8	NS			

(*Continued*)

TABLE 11.3

Prospective Studies Comparing Intracytoplasmic Morphologically Sperm Injection (IMSI) and Intracytoplasmic Sperm Injection (ICSI) According to Different ART Parameters (*Continued*)

		Comparison between IMSI and ICSI											
		Fertilization Rate (%)			Embryo Quality (% of Good Quality Early Cleaved Embryos)			Clinical Pregnancy Rate (*)/Cycle (%)			Miscarriage Rate (%)		
Studies	**Studied Population**	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***
Gonzalez-Ortega et al. (2012)[79]	60 couples Previous failure of > or = two ICSI cycles IMSI outcomes were matched with ICSI outcomes from similar couples Female age <38 years	89.0	91.2	NS	43.3	45.7	NS	50.0	63.0	NS	26.6	15.7	NS
Wilding et al. (2011)[51]	232 couples (110 ICSI, 122 IMSI) Infertility during 1–3 years Sperm concentration [1–20] millions/mL Exclusion: cryopreserved spermatozoa and surgical spermatozoa Female infertility factors	65.9	68.0	NS	(D3): 66.0	(D3): 98.6	0	(**) 40.0	(**) 65.6	0			
Wilding et al. (2011)[51]	Eight couples Couples with one previous ICSI failure IMSI was compared with the previous ICSI cycle	79.4	70.1	NS	60.3	83.6	S	0.0	37.5	NA			
Setti et al. (2011)[80]	500 couples (250 ICSI, 250 IMSI) Isolated male infertility, with altered semen parameters Exclusion: cryopreserved spermatozoa Less than 6 retrieved oocytes	78.9	79.2	NS	37.3	44.4	NS	36.8	37.2	NS	17.9	18.4	NS

Balaban et al. (2011)[81]	168 couples (87 ICSI, 87 IMSI) Male, female, or combined infertility	80.9	81.6	NS	(D3): 63.9	(D3): 66.4	NS	44.4	54.0	NS			
Knez et al. (2011)[88]	57 couples (37 ICSI, 20 IMSI) Male infertility, with altered semen parameters All arrested embryos following a prolonged 5-day culture in previous ICSI cycles	52.7	51.2	NS				8	25	NS			
Setti et al. (2012b)[82]	160 couples with IVF associated with preimplantation genetic screening for advanced maternal age (80 ICSI, 80 IMSI) Exclusion: severe sperm alterations Less than six retrieved oocytes	84	80	NS	48.5	54.5	NS	(***) 47.4	(***) 54.4	NS			
Cavagna et al. (2012)[86]	100 couples (50 ICSI, 50 IMSI) Group I: leukocytospermia semen samples with leukocyte count >1 × 10^6/mL (25 ICSI, 25 IMSI)	57.9	67.6	0.35				24	28	0.74	0	14.3	0.33
	Group II: Nonleukocytospermia semen sample (25 ICSI, 25 IMSI)	61.9	59.5	0.86				24	24	1	0	0	-
Knez et al. (2012)[75]	122 couples (70 ICSI, 52 IMSI) Isolated teratozoospermia							24	48	**<0.05**			
De Vos et al. (2013)[39]	350 couples Isolated male infertility Randomized oocytes Exclusion: cryopreserved spermatozoa and surgical spermatozoa Female infertility factors	77.3	79.1	NS	(D2): 38.5	(D2): 35	**0.047**	36.7	34.4	**NS**			

(*Continued*)

TABLE 11.3

Prospective Studies Comparing Intracytoplasmic Morphologically Sperm Injection (IMSI) and Intracytoplasmic Sperm Injection (ICSI) According to Different ART Parameters (*Continued*)

		Comparison between IMSI and ICSI											
		Fertilization Rate (%)			Embryo Quality (% of Good Quality Early Cleaved Embryos)			Clinical Pregnancy Rate (*)/Cycle (%)			Miscarriage Rate (%)		
Studies	**Studied Population**	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***	**ICSI**	**IMSI**	***p***
Marci et al. (2013)[83]	332 couples (281 ICSI, 51 IMSI) Fresh ejaculated spermatozoa	77.27	80.0	NS				30.96	33.3	NS	17.78	5.26	0.17
Setti et al. (2013)[76]	66 couples Couples undergoing ICSI as a result of advanced maternal age Female > or = 37 year old Male were normo-zoospermic patients Randomized study (33 ICSI, 33 IMSI)	69.5	67.2	NS	44.3	48.5	NS	13.8	60.0	**<0.001**	0.0	33.3	NS
El Khattabi et al. (2013)[84]	220 couples (130 ICSI, 90 IMSI) Couples with mild male factor who had at least two implantation failures after transfers of good quality embryos Male with teratozoospermia							26.0	24.0	NS			
Leandri et al. (2013)[72]	255 couples (139 ICSI, 116 IMSI) Male infertility $>3.10^8$ spermatozoa in the ejaculate $<1.10^8$ of motile spermatozoa recovered after density gradient Exclusion: female >39 years with day 3 follicle stimulating hormone level over 9 UI/L	63	56	**<0.05**				33	31	NS	30	27	NS
Delaroche et al. (2013)[63]	75 couples two previous IVF or ICSI failures	63.3	72.2	**0.02**	79.8	89.8	**0.009**						

Kim et al. (2014)[77]	66 couples 66 IMSI cycles were compared with the previous ICSI cycles Male OATS	65.0	67.7	NS	(D3): 46.8	(D3): 45.3	NS	12.5	33.3	**<0.05**	37.5	18.2	NS
La Sala et al. (2015)[73]	121 couples (73 ICSI, 48 IMSI) Inclusion: <4% of normal morphology $<1.5 \times 10^6$/mL activated spermatozoa after capacitation Exclusion: $<0.1 \times 10^6$/mL motile sperm	63.4	47.9	**0.001**	75.6	73.7	NS	20.5	22.9	NS	26.7	18.2	NS

*, Clinical pregnancy defined by a positive β-human chorionic gonadotrophonin (β-hCG) assay and presence of a fetal heartbeat by transvaginal ultrasound examination; **, pregnancy rate (positive β-hCG assay) per embryo transfer; ***, clinical pregnancy rate per embryo transfer; D2, embryo quality assessed on day 2; D3, embryo quality assessed on day 3; ART, assisted reproductive technology; IVF, in vitro fertilization; NA, not applicable; NS, not significant; OATS, severe oligoasthenoteratozoospermia; S, significantly different.

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12

The Usefulness of Antisperm Antibodies Testing

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Carolina Veaute, Rosa Molina, and Gustavo Luis Verón**

Introduction

Fertilization is a fundamental process that involves a coordinated sequence of interactions between two highly differentiated cells, the spermatozoon and the oocyte, giving rise to a diploid zygote. During this complex process, spermatozoa complete spermatogenesis and undergo several structural and functional modifications during epididymal transit, and, at ejaculation, sperm cells receive secretions from other male genital tract glands. During transport through the female reproductive tract, spermatozoa undergo a complex series of changes, collectively known as sperm capacitation, which are required to develop full fertilizing competence. On the other hand, the oocyte, which successfully completed morphogenesis in the ovary, is released in the oviduct at ovulation and meets spermatozoa at the fertilization site, in the ampulla. Spermatozoa interact with cumulus cells and the zona pellucida, undergo acrosomal exocytosis, and bind and fuse to the egg plasma membrane (oolemma). After syngamy, early embryonic development starts, and a few days later, the blastocyst is implanted in the uterus; embryogenesis and fetal development proceed until birth.[1–3]

The success of this enterprise may be challenged by numerous factors, among them antibodies that recognize sperm entities, called antisperm antibodies (ASA). This chapter has been organized into a group of sections. The section "Scientific and Biological Bases of Evaluation of ASA" presents basic concepts on ASA etiology in men and women and their impact on fertility. The section "Analytical Techniques to Measure the Presence of ASA" describes several analytical techniques to evaluate ASA presence and incidence for both women and men and presents data on ASA incidence retrieved from published reports. The section "ASA Evaluation in Men Attending an Andrology Laboratory: Our Experience" summarizes recent findings from our group on the assessment of sperm surface ASA in a large group of patients attending an Andrology Laboratory; findings on ASA incidence and their relationship with routine semen parameters and sperm kinematics are presented. The section "Laboratory Guidelines for ASA Assessment in Sperm and Biological Fluids" describes a set of practical guidelines to perform ASA evaluation, and provides information on commercially available kits as well. Lastly, the final section presents "Conclusions" of this chapter.

Scientific and Biological Bases of Evaluation of ASA

Etiology of ASA Development in Men and Women

To tolerate sperm antigens, the immune system exhibits a particular behavior, in both the male and the female reproductive tract. Sperm antigens arise in an individual's life long after the negative selection of self-reactive clones of T and B cells has occurred. Nevertheless, several mechanisms contribute to prevent the development of an immune response against sperm antigens. In men, autoimmunity against sperm is controlled mainly at the testis. The blood–testis barrier (BTB) is mainly formed by tight junctions between adjacent Sertoli cells, and it provides a physical separation between germ cells in the adluminal compartment of the tubule and the blood supply. Moreover, immune cells that are present in the interstitium have immunoregulatory properties, decreasing sperm antigen presentation and adaptive immune response induction (reviewed in Ref. 4).

In women, sperm antigen tolerance is accomplished by a complex series of mechanisms mainly driven by immunosuppressive factors of the seminal plasma (reviewed in Ref. 5) and by the activity of T regulatory cells in the female reproductive tract.[6]

Nevertheless, the genital tract is able to mount a protective immune response against pathogens.[7] Antibodies belonging to isotypes immunoglobulin G (IgG), IgA, and IgM are regularly present in genital tract fluids, and they come either from the plasma or from a local production. Although secretory IgA is the isotype typically produced at the mucosa, epithelial cells in the Fallopian tube and endocervix contained secretory component, suggesting the local production of secretory IgA[8] in female genital tract secretions, the predominant isotype being IgG.[9] It is accepted that IgG moves down a gradient from blood to the uterine lumen.[10] IgG and IgA concentrations are influenced by menstrual cycle stage and are directly regulated by the sex steroid hormones, i.e., progesterone and estradiol.[7,10,11]

Seminal plasma has a pronounced contribution of Igs from plasma. Relative distribution and molecular characteristics of Igs are similar to those found in plasma.[12] However, local production of antibodies is suggested by the presence of IgM, which does not transudate from plasma, and by the observation that in patients with ASA, sperm-associated and serum antibodies from the same patient could recognize different sperm antigens.[13,14]

Considering that immunocompetence of the genital tract and immunoregulation must coexist, it is likely that any breach of this balance may induce autoimmunity against sperm. In men, a genital tract obstruction has been associated with the presence of ASA, as reported in individuals with congenital bilateral absence of vas deferens (CBAVD) or cystic fibrosis (CF)[15–18] as well as in men with vas deferens obstruction caused by childhood herniorrhaphy[19] or with acute epididymitis.[20] The presence of ASA has also been reported in prepubertal boys with testicular failure,[21] in men with cryptorchidism[22,23] or testicular cancer,[24] or men subjected to testicular sperm extraction procedures,[25] although no clear relationship has been fully demonstrated in all cases. Physical damage of the BTB, as it occurs after testicular injury (testicular torsion, trauma, or surgery), may induce an inflammatory environment where sperm antigens are exposed together with danger signals, overcoming the natural regulatory conditions of the testis and leading to ASA production.[26–28] In this regard, a high incidence of ASA in men who have undergone vasectomy and later subjected to vasovasostomy has been documented.[29–31] Contrasting with these findings, ASA are often found in varicocele patients, but surgical repair of varicocele has not always been associated with reduced ASA levels.[32,33]

According to the mechanism proposed, infection is another condition that could simultaneously affect the BTB anatomic functionality and stimulate an inflammatory response. In this regard, primary or secondary autoimmune orchitis, an acute inflammatory reaction of the testes secondary to infection, is characterized by the presence of ASA.[34] In women, the presence of infection in the female genital tract has also been associated with the occurrence of ASA.[35–37] It has been proposed that a local inflammatory reaction would alter the presentation of sperm antigens, inducing an adaptive immune response against them, together with other immunopathological changes related to infertility.[38,39]

Both in men and in women, *Chlamydia trachomatis, Ureaplasma urealyticum,* and *Mycoplasma hominis* are among the most studied microorganisms related with the presence of ASA.[35,37,40–43] Cross-reactivity between sperm and bacteria antigens has been proposed as an alternative mechanism for the induction of ASA [37,41]. In fact, heat shock protein 70 (HSP70) from *C. trachomatis* and urease complex component (UreG) from *U. urealyticum* share epitopes with the sperm proteins HSPD1, HSPA2, and HSPA1L,[44] and nuclear autoantigenic sperm protein,[40] respectively. In addition, it was observed that patients with human papillomavirus (HPV) infection have a high incidence of ASA, and these antibodies mainly bind to spermatozoa containing viral proteins, suggesting that the presence of HPV components on the sperm surface could be an antigenic stimulus for ASA formation.[45] However, controversial results from different studies call into question the association of ASA with infection.[46,47]

Some evidence has led investigators to propose alternative hypotheses to explain the occurrence of ASA in women, although further studies are needed. The association of particular human leukocyte antigen (HLA) haplotypes with sperm-immobilizing antibodies might indicate that only a group of women are capable of mounting an immune response against sperm antigens.[48] The idiotype/anti-idiotype theory has been proposed to have a role in inducing ASA in women in response to ASA from their partners.[49] Another possible cause of ASA in women is based on the observation that antibody-coated

sperm can stimulate the production of interferon gamma (IFN-γ) by T lymphocytes from female donors, potentially inducing an inflammatory response that would lead to sperm immunity.[50] These mechanisms may explain the correlation observed between the occurrences of ASA in women and in their partners.[51]

ASA Effects on Fertility

The potential detrimental effect of ASA on male and female fertility was brought to the attention of reproductive biologists back in the 1950s. The studies conducted on rodents described the deleterious consequences of autoimmune responses to sperm and testis antigens, by producing orchitis and aspermatogenesis.[52] Their clinical relevance was provided first by a study describing spontaneous sperm agglutination of ejaculated spermatozoa in association to the detection of serum ASA in infertile men.[53] A few years later, a report described the presence of ASA in sera of women with unexplained infertility.[54]

Since then, numerous reports have related the detection of ASA in whole spermatozoa and in biological fluids with alterations in gametes and embryos and a consequent decreased conception rate in men and women. Figure 12.1 briefly summarizes some of the fertilization-related events in which ASA interference has been reported.[55] There is evidence indicating that agglutinating ASA reduce sperm

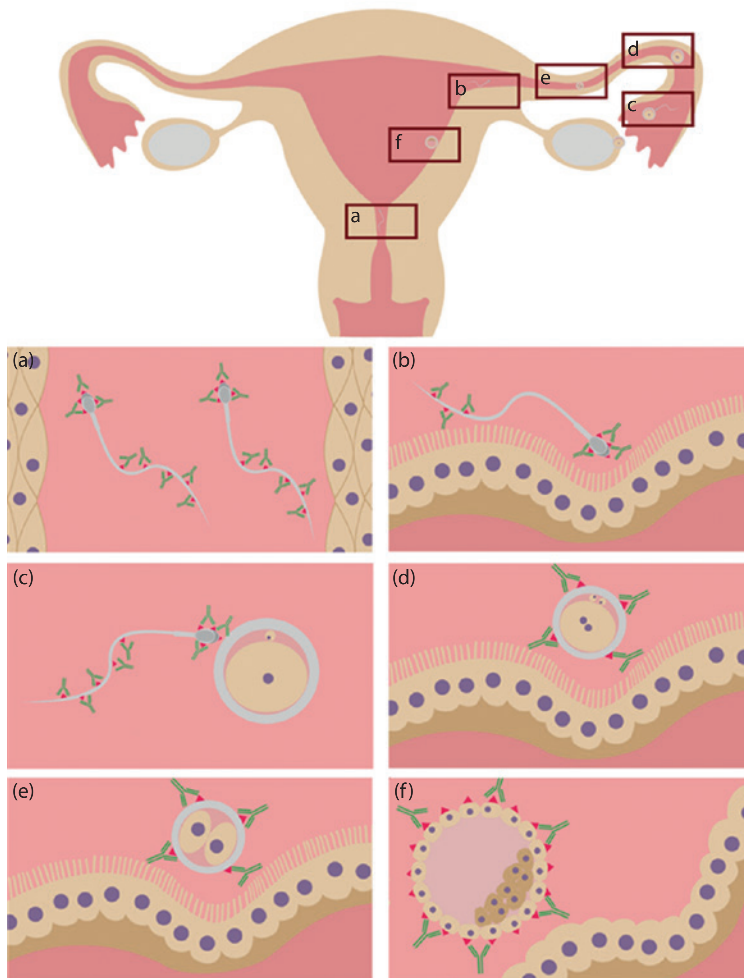

FIGURE 12.1 Steps of the fertilization process that can be affected by the presence of antisperm antibodies (ASA). ASA can affect (a) sperm passage through the cervical mucus, (b) sperm transport through the uterus, (c) sperm–oocyte interaction, (d, e) early embryo development, and (f) embryo implantation.

forward progressive motility and affect sperm penetration through cervical mucus[56] and transport to the Fallopian tubes.[57] In addition, there are reports describing ASA impairment of sperm capacitation,[58,59] as well as sperm interaction with oocyte vestments, in particular the zona pellucida [60] and the oolemma.[61] With regard to the ASA effect on acrosomal exocytosis, our group previously reported the presence of antibodies toward spermatozoa in follicular fluid with the ability to modulate the release of acrosomal contents, affecting sperm–zona pellucida interaction.[62]

The use of assisted reproductive technologies for infertility treatment led investigators to further assess the effect of male and female ASA on in vitro fertilization (IVF), early embryonic development, and pregnancy outcome. Our team previously reported the presence of ASA in sera from women undergoing IVF embryo transfer and their deleterious effect on IVF.[63] Moreover, sperm surface ASA were found to impair IVF, early embryonic development, embryo implantation, and fetal development.[64] Several other studies addressed this question, although results are still inconclusive. In this regard, a systematic meta-analysis study involving more than 4000 reproductive cycles (both IVF and intracytoplasmic sperm injection [ICSI]) revealed no relationship between semen ASA levels and pregnancy rates.[65] The discrepancies found may be attributed, at least in part, to patient heterogeneity in the study groups, as well as the methods and thresholds used for ASA assessment.

Analytical Techniques to Measure the Presence of ASA

Methods to Measure ASA in Spermatozoa and Fluids

ASA are mainly of Ig classes IgA and IgG, whereas IgM class antibodies are rarely found in semen. These antibodies can be detected on the sperm surface and/or they can be found free in the seminal fluid, in the male or female serum, in the cervical mucus, and/or in the follicular fluid.

Among assays developed to test the presence of ASA, two are currently the most used: the mixed antiglobulin reaction (MAR) test[66,67] and the immunobead binding (IB) test,[68,69] or their commercially available options. Both tests detect ASA on the surface of live spermatozoa by incubating motile spermatozoa with Ig-coated particles. In the presence of ASA, these particles adhere to the sperm surface; the percentage of motile spermatozoa with bound particles and their cell surface localization are recorded by observation under the microscope. Whereas the MAR test is performed on a fresh semen sample, the IB test requires semen centrifugation to remove the seminal plasma.

Using either method, the presence of ASA can be evaluated directly on the sperm cells (direct method) or in biological fluids (indirect method) after incubating them with donor ASA-free spermatozoa. As the first step, it is recommended to determine ASA on the sperm surface, and afterward, their presence in body fluids.[70] If there are insufficient motile spermatozoa to perform the direct test, indirect tests must be used because samples with poor motility may yield false-negative results. It is considered that high ASA titers in fluids are related to ASA bound to the sperm membrane, which may impair sperm performance. The World Health Organization (WHO) semen analysis manual (fifth edition) includes ASA determination as part of the basic semen evaluation, and the MAR and IB tests are detailed.[71]

The direct IgG and IgA MAR tests are performed by mixing fresh, untreated semen with latex particles (beads) or treated red blood cells coated with human IgG or IgA. A "bridging" antibody (anti-IgG or anti-IgA monospecific) is used to bring the antibody-coated beads into contact with spermatozoa carrying IgG or IgA. The formation of mixed agglutinates between beads and motile spermatozoa is indicative of IgG or IgA antibodies on the sperm surface (Figure 12.2). If spermatozoa do not present ASA on their surface, they will move freely, not covered with beads. Agglutinated beads will prove the reactivity of the particle antibodies and antiserum. In some cases, massive particle attachment might even cause sperm immobilization. The percentage of motile spermatozoa with bound beads/red blood cells is recorded.

In the direct IB test, spermatozoa must be devoid of seminal plasma by centrifugation. "Washed" spermatozoa are incubated with polyacrylamide beads (2–10 µm) coated with covalently bound rabbit antihuman Igs against IgG, IgA, and/or IgM, and the binding of beads to motile spermatozoa indicates the presence of Igs on the sperm surface (Figure 12.3).

In addition to the direct ASA tests, indirect tests are performed to evaluate the presence of sperm antibodies in body fluids, among them seminal plasma, blood serum, follicular fluid, and bromelain-solubilized

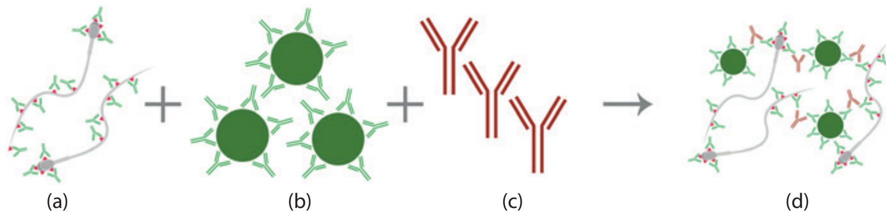

FIGURE 12.2 Schematic representation of the principle of the direct mixed antiglobulin reaction (MAR) test. (a) Fresh, untreated semen sample. (b) Latex beads or treated red blood cells coated with human immunoglobulin (Ig)G or IgA. (c) Anti-human IgG or IgA. (d) Mixed agglutinates composed of beads/red blood cells and motile spermatozoa (indicating the presence of IgG or IgA on the sperm surface). The percentage of motile spermatozoa with bound beads/red blood cells is recorded.

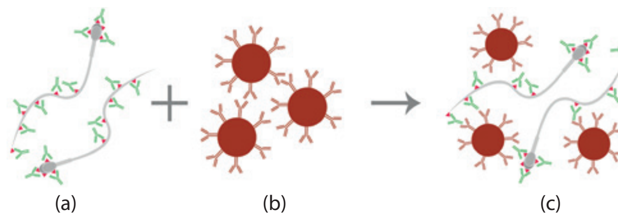

FIGURE 12.3 Schematic representation of the principle of the direct immunobead binding (IB) test. (a) Washed spermatozoa. (b) Polyacrylamide beads coated with rabbit antihuman immunoglobulin (Ig)G, IgA, and/or IgM. (c) Beads bound to motile spermatozoa (indicating the presence of IgG, IgA, and/or IgM on the sperm surface). The percentage of motile spermatozoa with bound beads is recorded.

cervical mucus. The diluted, heat-inactivated fluid suspected to have ASA is incubated with ASA-negative donor spermatozoa previously devoid of seminal plasma. Any ASA in the suspect fluid will bind specifically to the donor spermatozoa, which are then assessed in a direct test, as already described. For reliable results, it is important to allow sufficient time for the sperm–antibody interaction because it may take up to 10 minutes for the mixed agglutination to become visible. However, it should be considered that sperm motility declines with time, and the test results depend on the presence of motile spermatozoa. Both indirect MAR and IB tests can be performed (Figures 12.4 and 12.5). A description of the protocol to perform

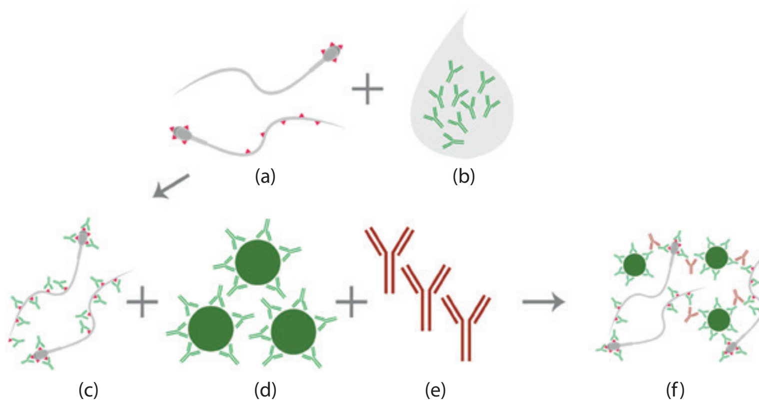

FIGURE 12.4 Schematic representation of the principle of the indirect mixed antiglobulin reaction (MAR) test. (a) Washed antibody-free donor spermatozoa. (b) Heat-inactivated fluid with antisperm antibodies (ASA). (c) Donor spermatozoa with bound ASA. (d) Latex beads or treated red blood cells coated with human immunoglobulin (Ig)G or IgA. (e) Anti-human IgG or IgA. (f) Mixed agglutinates composed by beads/red blood cells and motile spermatozoa (indicating the presence of IgG or IgA in the fluid). The percentage of motile spermatozoa with bound beads/red blood cells is recorded.

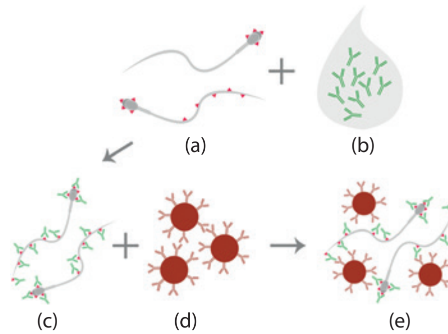

FIGURE 12.5 Schematic representation of the principle of the indirect immunobead binding (IB) test. (a) Washed antibody-free donor spermatozoa. (b) Heat-inactivated fluid with antisperm antibodies (ASA). (c) Donor spermatozoa with bound ASA. (d) Polyacrylamide beads coated with rabbit antihuman immunoglobulin (Ig)G, IgA, and/or IgM. (e) Beads bound to motile spermatozoa (indicating the presence of IgG, IgA, and/or IgM in the fluid). The percentage of motile spermatozoa with bound beads is recorded.

TABLE 12.1

Advantages and Disadvantages of the MAR Test and the IB Test

	Advantages	Disadvantages
MAR test	• It allows ASA evaluation on semen (direct test) and fluids (indirect test) • It is able to detect ASA isotype and location on the sperm surface • It is easy to perform • It can be performed on a fresh semen sample • It requires a small aliquot of semen sample (10 µL per determination) • It requires minimal equipment and technical expertise • It has good sensitivity and specificity • It is commercially available	• Beads form clumps
IB test	• It allows ASA evaluation on semen (direct test) and fluids (indirect test) • It is able to detect ASA isotype and location on the sperm surface • It is easy to perform • It requires minimal equipment and technical expertise • **It is precise, avoiding ASA masking by seminal plasma components** • It has good sensitivity and specificity • **It is commercially available**	• **It is time consuming** • **ASA titers present on the sperm surface or fluids cannot be determined** • **It requires a large volume of semen sample, with a higher concentration of motile spermatozoa than the MAR test** • It requires semen centrifugation to remove the seminal plasma

Abbreviations: MAR, mixed antiglobulin reaction; IB, immunobead binding; ASA, antisperm antibodies.

the MAR test (direct) and IB test (direct and indirect) following the guidelines from the WHO[71] is presented; moreover, information on the commercially available kits based on the MAR and IB tests is also included (see the section "Laboratory Guidelines for ASA Assessment in Sperm and Biological Fluids").

Both the MAR test and the IB test have several advantages and limitations. Table 12.1 summarizes some of these characteristics. Several studies have compared the sensitivity obtained with both

techniques but they do not always agree. The differences may be attributed to the type of method (direct or indirect) compared in each report.[72–75]

In addition to the MAR test and IB test procedures, the presence of ASA can be objectively evaluated using flow cytometry[76,77] and radiolabeled agglutinin assays.[76] For both techniques, a specific anti-Ig is labeled (with a fluorescent or radioactive marker, respectively) and mixed with the sperm sample. Flow cytometry allows the quantification of Ig level in live spermatozoa and can be coupled with immunocytochemistry to determine the localization of ASA on the sperm regions.

The enzyme-linked immunosorbent assay (ELISA) can also be used for auto- and iso-ASA-specific detection and quantification.[78] Anti-human Igs are covalently linked to an enzyme and added to fixed spermatozoa or to sperm extracts (previously incubated with the test fluid in the indirect assay). Antibody–enzyme Ig complexes are detected by a specific enzyme substrate, resulting in a color change that can be measured. The main disadvantage of this technique is that sperm fixation may disrupt plasma membrane, altering antigen detection.

The sperm agglutination tests (SAT) are able to detect the presence of multivalent ASA (mainly IgA and IgM) in serum or semen, with the ability of cross-linking several spermatozoa. Fluid samples are heat inactivated and serially diluted, motile spermatozoa from an ASA-negative donor are added, and sperm agglutination at each dilution is determined. The agglutination tests are known as the tray-slide agglutination test[54] or the tray agglutination test (TAT)[79] and the macroscopic gelatin agglutination test (GAT).[80] False-positive results can occur because bacteria or non-Ig proteins can also cause sperm agglutination. In addition, the sperm immobilization test (SIT)[81,82] is based on the principle that surface ASA can cause loss of sperm motility in the presence of complement. This assay uses patient serum mixed with motile spermatozoa and an external source of complement, and sperm immobilization is recorded. It is worth mentioning that SIT is not useful for the detection of ASA in samples with IgA because IgA does not fix complement.

ASA Incidence in Men and Women

Until the late 1970s, most laboratories performed assays to diagnose the presence of ASA by using sperm agglutination, sperm immobilization, or indirect immunofluorescence. The range of ASA-positive cases varied from 2% to more than 30%, with a large overlap between fertile and infertile groups.[83]

Since the publication of reports on the use of MAR and IB tests to assess ASA in the sperm surface or in male and female fluids, several publications described results on the incidence of ASA in patients consulting for infertility. Table 12.2 (section A) summarizes a total of nine studies identified in the literature in men from couples suspected or under treatment for infertility evaluated by means of the direct MAR or IB tests. From these results, sperm surface ASA incidence is estimated to be 9% (median value; range 5%–44%), depending on the study. In some cases, the studies listed included control groups, in which ASA incidence ranged from 0.9% to 6%. As shown, the sample number varied among studies (111–1060 men in the study group), as well as the cutoff value to define ASA-positive cases (10%, 20%, 40%, 50%). In addition, the results from a group of studies in which ASA were evaluated in patients diagnosed with pathologies in which ASA have been reported (among them male genital tract obstruction, varicocele, systemic autoimmune disease, testicular failure, and psychogenic anejaculation) are listed in Table 12.2 (section B).

Similarly, iso-antibodies toward sperm antigens have been detected in women. Studies have evaluated their presence mainly in serum; a summary of a group of studies found in the literature is presented in Table 12.3. From these results, female ASA-positive incidence range was 2.9%–64.4%. The studies included in this table involved evaluations done with other techniques in addition to indirect IB test.

Overall, this analysis revealed a great variability in the prevalence of iso- and auto-ASA. Further studies involving large population of patients, evaluated with standardized methods performed under strict quality control procedures, will help in the precise assessment of ASA incidence in individuals consulting for infertility.

TABLE 12.2

ASA Incidence in Men

A. Suspected of/under Treatment for Infertility

Incidence (ASA Positive)	Method	Cases/Sample	Comments	Reference
16% (infertile men) 2% (fertile men) ($p < 0.0001$)	Direct MAR test (50% cutoff)	N = 1060 normozoospermic subfertile men N = 107 fertile men	—	84
9.4%	Direct MAR test (20% cutoff)	N = 1228 men	—	85
6%	Direct MAR test (10% cutoff)	N = 650 semen samples from men consulting for infertility	—	86
15% (abnormal semen) 6% (normozoospermic)	MAR/IB test (10% cutoff)	N = 144 men from infertile couples N = 39 normozoospermic men	—	87
9%	Direct MAR test (20% cutoff)	N = 111 men treated for infertility	ASA inhibited IVF and early embryonic development IVF rate: ASA positive = 44.2%; ASA negative= 84.4% Early embryonic cleavage rate: ASA positive= 28.3%; ASA negative = 63.1% embryos with at least three blastomeres	62
6.2% (IgG; infertile men) 0.9% (fertile men; cutoff 40%) 12.9% (IgG; cutoff 10%)	Direct MAR test (10% and 40% cutoff)	N = 750 subfertile men (infertility 0.6–10 years in duration) N = 110 volunteers (history of previous fertility)	—	88
5%	Direct MAR test (40% cutoff)	N = 312 men consulting for infertility	—	67
44% (IgG and IgA)	Direct IB test (10% cutoff)	N = 120 infertile men with suspected autoimmunity to sperm	Sperm-bound Igs showed decreased sperm penetration into cervical mucus in 97.6% cases	89
7.8%	Direct IB test (IgG and/or IgA; 20% cutoff)	N = 813 men		90

B. With Other Pathologies

Incidence (ASA Positive)	Method	Cases/Sample	Comments	Reference
Male Genital Tract Obstruction				
33% (IgA or IgG class) (preoperative)	Indirect IB test	N = 27 vasectomized men undergoing vasectomy reversal	An increase ($p < 0.05$) in postoperative incidence of ASA in men who achieved patency (27/45, 60%) No increase in men without spermatozoa in semen (4/10, 40%)	31
35% (on epididymal sperm) 16% (in epididymal fluid) 29% (in serum)	Direct and indirect IB test	N = 46 men with CBAVD	No statistical difference observed in the overall fertilization rate between the two groups Five pregnancies (31%) occurred in the ASA-positive group, of which two had sperm binding of 100% for IgG (all over sperm surface) and 90% (midpiece, tail) and 50% (tail, tail tip), respectively, for IgA Five pregnancies (18%) obtained in the negative group	15
Varicocele				
33%	MAR test (IgG; cutoff 10%)	N = 99 patients with varicocele	Response to varicocelectomy: improvement in ASA positive in patients with higher varicocele grade (median 2 vs. 1; $p < 0.05$) and lower ASA level (MAR-IgG = 48% vs. 92%; $p < 0.01$)	33
26.5% (men with varicocele) 38.5% (men without varicocele)	MAR test	N = 66 patients with varicocele. N = 84 men without varicocele.		92
26%	MAR test (direct, IgA and IgG; cutoff >10% and <40%)/("low positivity")	81 infertile men undergoing microsurgical inguinal varicocelectomy	Six months after surgery, ASA titer was reduced in 18 and increased in 3 cases	32
28% 0% (normal fertile men)	IB test	32 infertile men undergoing varicocele ligation	No changes in ASA after varicocele repair	93
Other Pathologies				
7.1% (men with autoimmune disease) 0% (controls)	Indirect IB test	N = 70 men with systemic autoimmune diseases. N = 80 healthy controls.		94
39% IgG + 7% IgA in adults 100% (children) 0% (controls) (IgG, IgA, IgM)	Indirect IB test IgG, IgA, IgM in (serum)	N = 183 infertile men with a history of cryptorchidism and/or inguinal hernia N = 82 prepubertal boys		95
31%	Direct and indirect IB test (serum)	N = 16 men with psychogenicanejaculation	The majority of ASA were directed against the sperm heads Surface ASA were mainly IgA isotype whereas serum antibodies were IgG isotype.	96

(Continued)

TABLE 12.2

ASA Incidence in Men (*Continued*)

B. With Other Pathologies

Incidence (ASA Positive)	Method	Cases/Sample	Comments	Reference
		Other Pathologies		
7% (indirect IB test) 48% (flow cytometry) 32% (ELISA) 65% (immunoblotting reactions with glycosylated antigens) 70% (with deglycosylated antigens)	Indirect IB test, flow cytometry, ELISA (serum), Western blotting (sperm extracts of glycosylated/ deglycosylated solubilized membrane antigens)	*N* = 69 prepubertal boys with testicular failure *N* = 7 healthy prepubertal boys		21

Note: The information listed was obtained from the abstract of the publication in most of the cases.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ASA, antisperm antibodies; Ig, immunoglobulin; MAR, mixed antiglobulin reaction; IB, immunobead binding; CF, cystic fibrosis; CBAVD, congenital bilateral absence of vas deferens.

TABLE 12.3

ASA Incidence in Women

Incidence (ASA Positive)	Method	Cases/Sample	Comments	Ref.
6.0% (3/50)	Antibody latex agglutination tests.	*N* = 50 women with unexplained infertility *N* = 44 women with a known cause of infertility Serum samples for antibodies detection Case–control study	All cases were seronegative for anti-*C. trachomatis* IgM and were PCR negative	97
51%	ELISA (total Ig)	*N* = 43 women after failure of ART		98
62.2% (infertile women) 3.3% (control) (indirect sperm agglutination test between sera and cervicovaginal secretions) 64.4% (infertile women) 3.3% (control) (indirect immunofluorescence test)	Radial immune diffusion. (immunoglobulins serum levels: IgG, IgA, IgM) Indirect immune fluorescence test Direct sperm agglutination test: ASAs detected in cervicovaginal secretion	*N* = 45 infertile women consulting for infertility *N* = 30 fertile women (control group)	42.8% cases presented head-to-head agglutination	99

Incidence (ASA Positive)	Method	Cases/Sample	Comments	Ref.
2.9% (8/273) (infertile women) 6.4% (5/78) (cases with past *C. trachomatis* infection) 1.5% (3/195) (cases without past *C. trachomatis* infection) ($p = 0.031$ between both groups).	SIT (serum)	$N = 273$ infertile women		35
20% (36/179)	Indirect IB test (cutoff 25%)	$N = 179$ women consulting for infertility		100
13.9 % (ELISA) 10.1% (SIT)	ELISA and SIT (serum)	$N = 158$ infertile women		101
21.6% 29.6% of the CM samples from 459 women	Sperm-immobilized activity	$N = 698$ infertile couples	Reduced sperm penetration of CM was significantly associated with serum titers of antisperm antibodies in both sexes and also with immobilizing activity in CM of women	102

Note: The information listed was obtained from the abstract of the publication in most of the cases.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; ASA, antisperm antibodies; Ig, immunoglobulin; SIT, sperm immobilization test; IB, immunobead binding; ART, assisted reproductive technology; CM, cervical mucus.

ASA Evaluation in Men Attending an Andrology Laboratory: Our Experience

Assessment of ASA Incidence and Relationship with Routine Semen Parameters and Sperm Kinematics in a Large Population of Patients

The presence of ASA has been negatively related to some routine semen parameters. Recently, a meta-analysis including more than 1000 cases was published.[103] These studies were conducted following 1999 WHO criteria and using the WHO manual for semen assessment. However, to date, no report on ASA testing conducted following the guidelines from the WHO manual released in 2010 (fifth edition) exists. The current manual is the first in using lower reference limit (LRL) values to define normality in each parameter (1.5 mL for semen volume; 15 million/mL for sperm concentration; 39 million for sperm count; 32% for sperm progressive motility; 58% for sperm vitality and for hypo-osmotic swelling (HOS) test score; 4% for sperm morphology; 5 million/mL for round cells; and 1 million/mL for peroxidase-positive cells).

Our group has recently completed a retrospective analysis in a large group of more than 7000 men subjected to routine semen analysis, and ASA evaluation (direct SpermMAR™ [IgG] test) following the guidelines described in the 2010 WHO manual.[104] The study was conducted to determine the incidence of sperm surface ASA and the association between ASA and semen parameters. Semen evaluation also included computer-assisted sperm analysis (CASA), a procedure that objectively evaluates sperm kinematics; in this regard, information on the impact of sperm-surface autoantibodies on sperm kinematics is scarce and inconclusive. For CASA, curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s), average path velocity (VAP; μm/s), linearity (LIN; arbitrary units, expressed as percentage), amplitude of lateral head displacement (ALH; μm/s), straightness (STR; arbitrary units, expressed as percentage), beat/cross frequency (BCF; Hz), mean angular displacement (MAD; degrees), and Wobble (WOB; arbitrary units, expressed as percentage) were evaluated in more than 2800 samples.

From a total of 7492 men included in the study, a 2.6% and 5.9% incidence of ASA-positive cases was found (cutoff 50% and 10%, respectively). In men having normal sperm concentration, motility, and morphology (normozoospermic; $n = 4593$ cases), ASA incidence was lower ($p < 0.05$) than that in the unselected population (2.0%, cutoff 50%; 4.9%, cutoff 10%).

When sperm parameters evaluated in 9482 semen samples were compared between ASA-positive and ASA-negative samples (cutoff 50%), a lower ($p < 0.0001$) sperm concentration, count, motility, and HOS test score in ASA-positive samples were found ($p < 0.0001$). Moreover, results of these parameters negatively correlated with ASA levels ($p < 0.0001$); a negative association between levels of sperm surface ASA and some sperm characteristics was found. Samples from the whole population depicting normal sperm concentration, motility, and morphology (normozoospermic) also had lower HOS scores in the ASA-positive group than those in the ASA-negative group ($p < 0.0001$). Moreover, HOS results did not correlate with sperm vitality in normozoospermic samples with high ASA levels (cutoff 50%), suggesting that ASA may affect sperm membrane integrity rather than cell viability. A diagram summarizing these results is presented in Figure 12.6.

Because sperm progressive motility was found significantly reduced in ASA-positive samples, it was of interest to further evaluate the relationship between ASA and motility characteristics by means of CASA. Evaluation of sperm kinematic characteristics in a subgroup of 2838 samples revealed lower ($p < 0.0001$) scores for VSL, LIN, BCF, and WOB in the ASA-positive (50% cutoff value) subgroup. In addition, VAP, ALH, and STR values were lower in the subgroup carrying antibodies, although with less significance. Interestingly, when results of CASA in samples depicting normal sperm concentration, motility, and morphology with or without ASA were compared, no significant differences were found for sperm kinematic parameters between groups despite the high levels of ASA found in ASA-positive samples. Findings suggested that these evaluations could not indirectly anticipate presence of clinically relevant ASA levels. Figure 12.7 shows a diagram summarizing these results.

Altogether, results from our recent work are in favor of performing ASA evaluation as a part of a thorough basic routine semen examination. Abnormally high levels of ASA were related to other sperm parameters that compromise sperm performance. Moreover, ASA may still be present in high levels in samples depicting routine sperm parameters within normal values and sperm kinematic values comparable to those observed in ASA-negative samples.

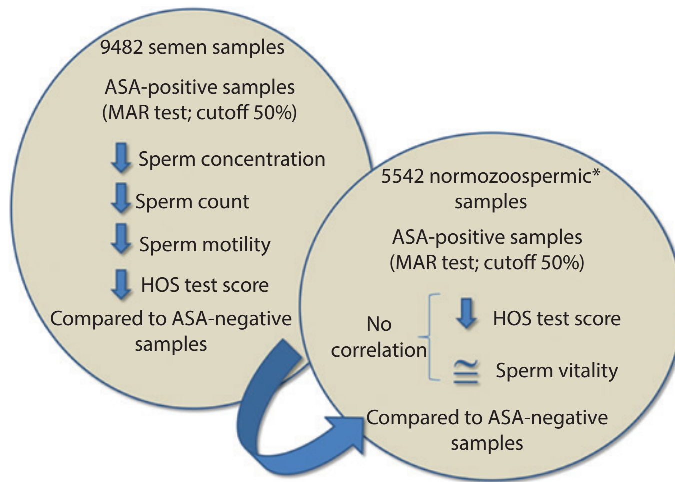

FIGURE 12.6 Antisperm antibodies (ASA) and routine semen parameters. Diagrammatic representation of results on the relationship between ASA levels and routine semen parameters in a large group of samples from men attending an Andrology Laboratory, and in a subgroup of normozoospermic samples. *Normal sperm concentration, motility, and morphology (WHO 2010 criteria). (From Verón GL et al., *Am J Reprod Immunol,* 76, 59–69, 2016.)

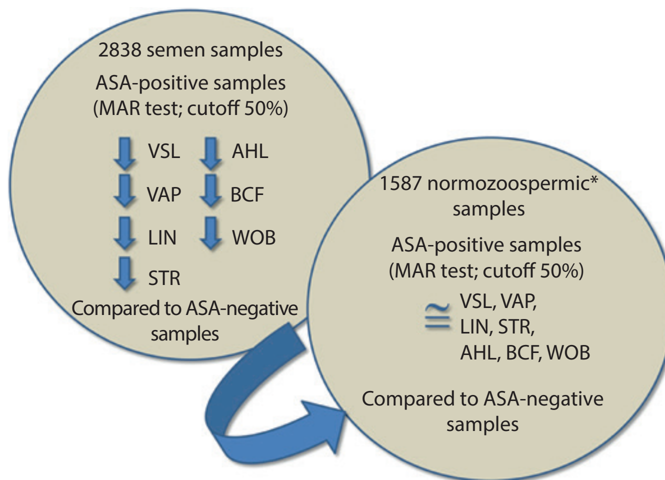

FIGURE 12.7 Antisperm antibodies (ASA) and routine sperm kinematics. Diagrammatic representation of results on the relationship between ASA levels and sperm kinematic parameters in a large group of samples from men attending an Andrology Laboratory, and in a subgroup of normozoospermic samples. *Normal sperm concentration, motility, and morphology (WHO 2010 criteria). (From Verón GL et al., *Am J Reprod Immunol,* 76, 59–69, 2016.)

Laboratory Guidelines for ASA Assessment in Sperm and Biological Fluids

The Direct MAR Test

Reagents Needed

Latex particles (beads) or treated red blood cells coated with human IgG or IgA

- Monospecific anti-human IgG or anti-human IgA

Procedure (WHO Manual, 2010)

- Mix the semen sample thoroughly and remove two aliquots of 3.5 μL semen. Place them on separate microscope slides.
- Add 3.5 μL IgG-coated latex particles (beads) to each droplet of semen and mix with the pipette tip.
- Add 3.5 μL antiserum against human IgG to each semen–bead mixture and mix with the pipette tip.
- Cover the suspension with a coverslip (22 mm × 22 mm) to provide a 20-μm-depth preparation.
- Maintain the slide horizontally for 3 minutes at room temperature in a humid chamber (e.g., on water-saturated filter paper in a closed Petri dish or other container).
- Examine the wet preparation with a microscope (preferentially equipped with phase-contrast optics) at ×200 or ×400 magnification. Repeat the observation after 10 minutes.
- Repeat the procedure using IgA- instead of IgG-coated beads and anti-IgA instead of anti-IgG antibodies.

Scoring

If spermatozoa have antibodies on their surface, the latex beads will adhere to them, so the percentage of motile spermatozoa with attached beads is recorded. Initially, the motile spermatozoa will move with few particles attached to them, but the binding increases with time. ASA-free spermatozoa will swim freely between the particles. Eventually, the agglutinates become so massive that the movement of the spermatozoa is severely restricted. Special attention must be paid to spermatozoa with nonprogressive motility that are close to beads, but are not attached to them. If there is doubt whether sperm have bound beads or not, the coverslip can be gently touched with a pipette tip.

- Score only motile spermatozoa that have two or more latex particles attached to them.
- Evaluate at least 200 motile spermatozoa in each replicate.
- Calculate the percentage of motile spermatozoa with attached beads.
- Record the class (IgG or IgA) and the latex particle binding site (sperm head, midpiece, principal piece). Ignore beads binding to tail-tip because this kind of ASA has not been associated with impaired sperm function.[105,106]

Quality Control

Include one slide with 3.5 μL ASA-positive semen and one with 3.5 μL ASA-negative semen as controls in each direct test. These semen samples should be from men with and without ASA, respectively, as detected in previous direct MAR tests. ASA-positive spermatozoa can also be produced by incubating an ASA-negative semen sample with an ASA-positive serum (see the section “The Indirect IB Test”).

Interpretation Guidelines

The WHO retains the consensus cutoff value of 50% motile spermatozoa with adherent particles. However, if the result is less than 50% motile spermatozoa with bound particles, the clinician should determine whether the presence of ASA is clinically relevant.

The Direct Immunobead Binding Test

Reagents Needed

- Dulbecco's phosphate-buffered saline (PBS) supplemented with bovine serum albumin (BSA) or Tyrode's–BSA solution:
  - Buffer I: 0.3% (w/v) BSA in Dulbecco’s PBS or Tyrode’s medium.
  - Buffer II: 5% (w/v) BSA in Dulbecco’s PBS or Tyrode’s medium.

- Both solutions must be sterilized by filtration through 0.45 μm and warmed to 25–35°C before use.
- Immunobeads (BioRad, Hercules, California) were prepared as follows:
  - Place 0.2 mL stock bead suspension in a centrifuge tube and add 10 mL buffer I.
  - Centrifuge at 500*g* or 600*g* for 5–10 minutes.
  - Discard the supernatant and resuspend the beads in 0.2 mL buffer II.

Procedure (WHO, 2010, with Modifications)

- Mix the semen sample well, transfer the semen to a centrifuge tube, and make up to 10 mL with buffer I.
- Centrifuge at 500*g* for 5–10 minutes.
- Discard the supernatant and gently resuspend the sperm pellet in 10 mL fresh buffer I.
- Centrifuge again at 500*g* for 5–10 minutes.
- Discard the supernatant and resuspend the sperm pellet in 0.2 mL buffer II.
- Place two droplets of 5 μL washed sperm suspension being tested on different microscope slides.
- Add 5 μL immunobead suspension beside the sperm droplet and mix by stirring with the pipette tip.
- Place a 22 mm × 22 mm coverslip over the mixed droplet to provide a 20-μm-depth preparation.
- Store the slides horizontally for 3–10 minutes at room temperature in a humid chamber.
- Examine the slides with a microscope (preferentially equipped with phase-contrast optics) at ×200 or ×400 magnification.

Scoring

- Score *only motile spermatozoa* that have one or more beads bound. Record the site of beads binding, but ignore tail-tip binding.
- Evaluate at least 200 motile spermatozoa.
- Calculate the percentage of motile spermatozoa with attached beads.

Quality Control

In each test, ASA-positive and ASA-negative spermatozoa must be included as controls. Semen should be from men with and without ASA, respectively, as detected in previous direct IB tests. Alternatively, ASA-positive spermatozoa can be produced by incubation of a negative sample with an ASA-positive serum (see the section "The Indirect IB Test").

Interpretation Guidelines

The WHO retains the consensus cutoff value of 50% motile spermatozoa with bound beads. If the result is less than 50% motile spermatozoa with bound beads, the clinician should determine whether the presence of ASA is clinically relevant.

The Indirect IB Test

Procedure (WHO, 2010, with Modifications)

- Prepare the immunobead reagents and wash the donor spermatozoa as detailed for the direct assay.
- Prepare the fluid to be tested. If it is cervical mucus, prepare 10 IU/mL bromelain, dilute 1 + 1 (1:2) with 10 IU/mL bromelain, stir with a pipette tip, and incubate at 37°C for 10 minutes.

When liquefaction is complete, centrifuge at 2000*g* for 10 minutes. Use the supernatant immediately for testing or freeze at –70°C.

Inactivate any complement in the fluid to be tested (solubilized cervical mucus, serum, or seminal plasma) by heating at 56°C for 30–45 minutes.

- Dilute the heat-inactivated sample 1 + 4 (1:5) with buffer II (e.g., 10 μL fluid to be tested with 40 μL buffer II).
- Mix 50 μL washed donor sperm suspension with 50 μL of 1 + 4 (1:5) diluted fluid to be tested.
- Incubate at 37°C for 1 hour.
- Centrifuge at 500*g* for 5–10 minutes.
- Discard the supernatant and resuspend the sperm pellet in 10 mL fresh buffer I.
- Centrifuge again at 500*g* for 5–10 minutes.
- Discard the supernatant and repeat the centrifugation step.
- Gently resuspend the sperm pellet in 0.2 mL buffer II.
- Perform the IB test, as already described, with the donor spermatozoa preincubated with the fluid.
- Score and interpret the test as already described.

Quality Control

Include ASA-positive and ASA-negative samples, e.g., serum from individuals with and without ASA, respectively, as controls in each indirect test. Commercial ASA-positive and ASA-negative serum can also be used (see the section "Commercially Available Reagents").

Commercially Available Reagents

Kits that Follow the MAR Test Principle

Among the commercially available reagents to evaluate the presence of ASA that follow the MAR test principle, the most used are the SpermMAR™ and the MarScreen™ assays. A brief description is presented as follows.

SpermMAR™ (FertiPro N.V., Beernem, Belgium)

This assay allows the detection of IgG and IgA antibodies. The kit provides either IgG- or IgA-coupled latex particles and its corresponding antiserum. Despite its similarity to the MAR test, some modifications need to be done, as follows:

1. For the direct assay, 10 μL semen is mixed with 10 μL SpermMAR™ latex particles and with 10 μL antiserum.
2. For the indirect test, manufacturers suggest the use of motile spermatozoa from an ASA-negative donor, selected by the swim-up technique. A 50-μL aliquot of the sperm suspension is mixed with 50 μL inactivated fluid to be tested (previously diluted 1/16) and incubated for 1 h. The spermatozoa are mixed with the particles and the antiserum, as for the direct assay, and the percentage of motile spermatozoa carrying latex particles is evaluated. It is suggested to use the cutoff of 40% spermatozoa with bound particles for both the direct and the indirect assays; when 10%–39% of the motile spermatozoa carry particles, immunological infertility is suspected and additional tests should confirm the diagnosis. Manufacturers suggest carrying out first the IgG test, and when a positive result is obtained, they indicate to perform the IgA test.

This test has been widely used in the last 25 years to evaluate the presence and effect of auto- and iso-antisperm antibodies.[107] We have recently used this assay to assess the incidence of surface sperm autoantibodies in a large population (see the section “ASA Evaluation in Men Attending an Andrology Laboratory: Our Experience”).

MarScreen™ (Bioscreen, Origio, Måløv, Denmark)

This kit provides color latex beads, which helps in the identification of the Ig type present on the sperm surface. Blue latex beads are conjugated to human IgG, whereas red and green beads are conjugated to human IgA and IgM, respectively.

1. In the direct assay, 10 μL semen is mixed with 10 μL MarScreen™ beads and with 10 μL antiserum; after 1 minute the slide is evaluated.
2. The protocol for the indirect assay is similar to that of the SpermMAR™ test, but in this case, washed spermatozoa (at least 10 million motile sperm/mL) can be used.

MarScreen™ has been used to analyze the relationship between the presence of ASA, sperm movement, and semen quality,[87] as well as reproductive outcome after IVF.[108]

Kits that Follow the IB Test Principle

Immunospheres® (BioScreen) is a method for the detection of ASA that follows the same principle of the IB test (beads coupled with anti-human Ig), but using color-coded beads. There are two kits: (1) one is composed of white latex beads coupled with goat anti-human heavy and light chains, which allow the detection of any Ig present on the sperm surface; and (2) the other is composed of red latex beads coated with goat anti-human IgA, blue beads with anti-human IgG and green beads with anti-human IgM, which specifically detect IgA, IgG, and IgM, respectively. Manufacturers suggest first using anti-IgA beads, and then performing the assay with anti-IgG and anti-IgM beads.

Contrasting with the IB, Immunospheres® do not tend to clump. All beads have a uniform size of 3 μm. Moreover, colored beads may be easily detected by light microscopy. For the indirect method the manufacturers suggest mixing 50 μL tested serum with 400 μL medium and 50 μL donor sperm suspension (at least 50 million motile sperm/mL), and incubating them for 1 hour. The results obtained using Immunospheres® were similar to those of the IB test.[109]

Human Sera

Positive and negative sera to include as control in ASA testing are commercially available. SpermMAR™ (FertiPro N.V.) provides IgG-positive and IgG-negative sera with ASA levels higher than 80% and lower than 20%, respectively. Sperm antibody A/G sera (BioScreen Inc.) can be used at different dilutions with most of the described tests.

Conclusions

We have presented a brief summary on ASA etiology and the effects of iso- and auto-antibodies on fertility (Figure 12.1). In addition, we have detailed the methodologies used to assess the presence of ASA on spermatozoa and biological fluids (Figures 12.2 through 12.5, Table 12.1). Moreover, we have provided a list of studies on ASA incidence in men and women suspected of infertility or affected by certain pathologies (Tables 12.2 and 12.3). Finally, we have summarized a study conducted by our group on a large population of men attending an Andrology Laboratory, an investigation in which ASA incidence and impact on routine semen parameters and sperm kinematics were determined (Figures 12.6 and 12.7).

A flow diagram showing the basic concepts included in this chapter is depicted in Figure 12.8.

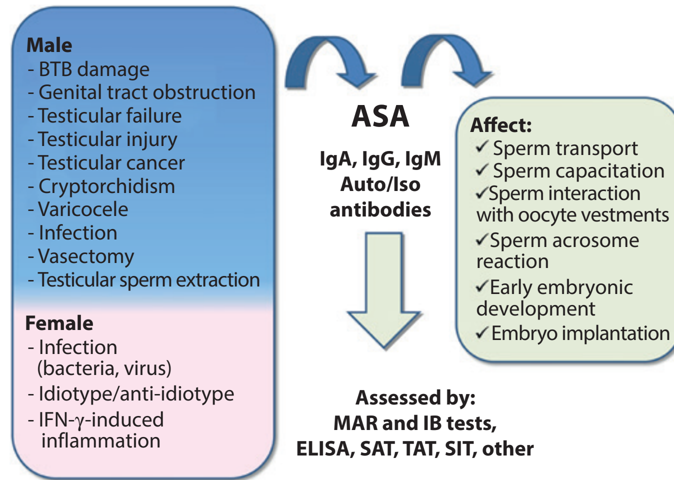

FIGURE 12.8 A scheme on antisperm antibodies (ASA) usefulness. Flow diagram depicting ASA etiology (left), ASA types and available methods for ASA evaluation (center), and ASA effects on fertilization-related events (right).

Altogether, the studies summarized in this chapter lead us to conclude the following:

- ASA testing is useful because assays can specifically identify antibodies present in the surface of spermatozoa and in biological fluids.
- ASA testing is useful because ASA detected on the sperm surface were found to be associated with abnormalities in some semen parameters (i.e., sperm concentration, count, motility, HOS test score, and sperm kinematic characteristics).
- ASA testing is useful because antibodies toward spermatozoa may be present in semen samples depicting normal sperm concentration, motility, morphology, and sperm kinematics and may affect sperm functionality.
- ASA testing is useful because it may help the physician in the identification of male/female pathologies of the reproductive tract (i.e., male genital tract obstruction, inflammation, trauma; and male and female genital tract infection).
- ASA testing is useful because antibodies may affect sperm performance in fertilization, embryonic development, and implantation.
- ASA testing is useful because results obtained may help the physician selecting the appropriate procedure to treat infertility.

However, it is highly relevant to consider the following:

- ASA testing requires the use of an adequate assay. In this regard, the selected test depends on the type of sample to evaluate, as recommended in the 2010 WHO manual for semen evaluation.
- ASA testing must be validated with proper positive and negative controls and quality control procedures.
- ASA testing must be performed by well-trained professionals.
- ASA testing may, in some cases, provide better results if a combination of suggested assays are applied to the same evaluated sample.

In the future, controlled-design prospective studies with defined set of patients (defined clinical diagnosis) involving standardized methodologies for ASA assessment (method and cutoff levels), ART procedures (IVF and ICSI defined protocols), and endpoint determinations (i.e., fertilization rate,

implantation, and pregnancy rates) will help determine ASA incidence in men and women suspected of infertility as well as individuals with other pathologies.

In any case, there is still a long way to go in the field of ASA testing. Current assessment is based on the detection of Igs bound to the sperm surface or present in a biological fluid that recognizes a sperm antigen. However, it does not distinguish whether the Igs bind to a specific antigen from an entity that plays a key role in sperm function or if it is rather irrelevant for an adequate sperm gamete performance. Many efforts have been devoted to identify sperm entities involved in sperm function to define targets to which antibodies may produce any harm. The development of ASA tests for sperm-specific antigens related to the male gamete functions may complement current procedures and provide more accurate information on the potential effect of ASA on fertility.

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13

The Measurement of Oxidative Stress in Semen and Use in Assisted Reproduction

Ashok Agarwal, Joseph Vitale, and Anthony Kashou

Summary

Infertility has become a major clinical problem, affecting 15% of all couples worldwide. Almost 25%–40% of the infertility cases are attributed to male factor. Among the numerous factors that contribute to infertility, oxidative stress (OS) has been shown to play a major role. OS occurs when the levels of reactive oxygen species (ROS) exceed the seminal antioxidant capacity. Unstable free radicals or ROS can cause cellular damage leading to sperm dysfunction, abnormal semen quality, and compromised fertility. Hence, detection of these seminal oxidants is of great concern and is possible by a variety of techniques used in andrology and infertility clinics. This chapter discusses the use and efficacy of such techniques in measurement of ROS and OS.

Introduction

Semen analysis has consistently demonstrated the ability to predict the fertility status of a man. Reduced semen parameters—such as sperm concentration, motility, and morphology—have been commonly found in subfertile men.[1] A significant factor implicated in the decline of semen quality is oxidative stress (OS), which results due to an excess production of reactive oxygen species (ROS) and/or a reduced total antioxidant capacity (TAC).[2] The imbalance between ROS levels and antioxidants demonstrates harmful effects on cell function.[3]

Reactive Oxygen Species

ROS represent a group of unstable and highly reactive molecules that lack a fully paired outer electron shell. Hence, ROS are often interchangeably termed free radicals and are produced during normal cellular metabolism. Spermatozoa and seminal leukocytes are the two main sources of free radicals within semen. The physiological role for ROS in regulation of normal sperm functions such as capacitation, acrosome reaction, and signaling processes ensures that fertilization has been well documented.[4,5] However, their extreme volatility gives way to toxic and pathological effects if not kept at low and controlled levels. Spermatozoa are even more vulnerable to ROS damage due to the lack of a full antioxidant repertoire. This can compromise the sperm's ability to carry out its normal physiological functions, and hence, contribute to infertility.[2]

Oxygen-derived free radicals represent the majority of ROS, acting as powerful oxidants. Superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) are among the potent ROS, with the hydroxyl radical having the greatest potential for deleterious effects. These highly reactive molecules have the ability to oxidatively alter cell structure and function. Disruption of membrane permeability leaves the cell susceptible to irreversible damage (Figure 13.1, Table 13.1).

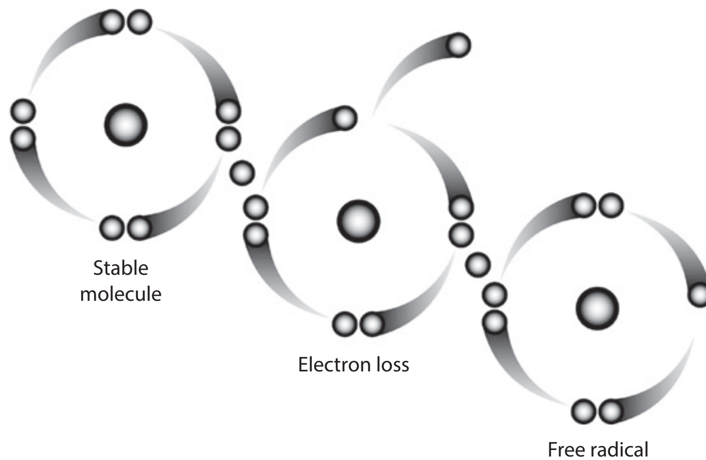

FIGURE 13.1 Generation of free radicals.

TABLE 13.1

Comprehensive List of ROS and RNS

Reactive Oxygen Species (ROS)	Symbol	Reactive Nitrogen Species (RNS)	Symbol
Hydroxyl	OH^-	Nitrous oxide	NO^-
Superoxide	O_{2-}^{-}	Peroxynitrate	$OONO^-$
Nitric oxide	NO^-	Peroxynitrous acid	ONOOH
Peroxyl	RO_2^-	Nitroxyl anion	NO^-
Lipid peroxyl	LOO^-	Nitrogen dioxide	NO_2^-
Peroxynitrate	$ONOO^-$	Dinitrogen trioxide	N_2O_3
Hydrogen peroxide	H_2O_2	Nitrous acid	HNO_2
Singlet oxygen	$^{-1}O_2$	Nitryl chloride	NO_2Cl
Hypochloric acid	HOCl	Nitrosyl cation	NO^+

Counteracting Reactive Oxygen Species

Fortunately, normal physiological defense mechanisms exist endogenously. Cells contain a host of antioxidants that counteract oxidant effects. Both enzymatic and nonenzymatic antioxidants act in concert to prevent OS from ensuing. Human gametes possess a variety of antioxidants to combat ROS. Seminal fluid contains enzymatic antioxidants—superoxide dismutase (SOD), catalase, and glutathione peroxidase—which are capable of catalyzing the production of nonharmful, reduced byproducts. These enzymes are essential in neutralizing and reversing the potentially toxic effects of ROS. In fact, both enzymatic and nonenzymatic (e.g., vitamins C and E) antioxidants have demonstrated the ability to improve semen quality.[6,7]

An Inadequate Balance

An ideal balance of oxidants and antioxidants does not always exist. Oxidant overload and/or diminished TAC can result in OS. In addition, cytoplasmic scavenging enzymes in spermatozoa are often low, owing to their extreme susceptibility to oxidative damage. A sudden shift to an oxidative state may result in impaired motility, premature acrosomal reaction, lipid peroxidation, DNA damage, and eventually apoptosis.[3,8] Hence, this delicate balance of oxidants and antioxidants becomes critical in maintaining proper sperm structure and reproductive function in assisted reproductive technology (ART).

Oxidative Stress in the ART Setting

Over the years, ARTs have become the treatment of choice for male and female infertility. Yet, despite numerous advances, success rate in ART remains unsatisfactory due to several implicated factors.[9–11] Among such factors, OS has emerged as one of the most important issues affecting ART outcome.[12,13] Elevated levels of ROS in OS have been noted to induce membrane and DNA damage, effectively reducing both sperm quality and the potential for successful in vitro fertilization (IVF).[14,15] ART involves a sequence of in vitro procedures that naturally create a hostile environment, in which hyperoxic conditions impair the ability of the spermatozoa to carry out their typical in vivo function. Normal in vivo defense mechanisms in spermatozoa become strained once semen is exposed to the external environment. This additional source of ROS makes the already-antioxidant deficient spermatozoa even more vulnerable to OS. Hence, the oxygen-rich atmosphere places enormous pressure in maintaining the functional–fertilization capacity of sperm during assisted reproduction. Thus, OS plays a significant role in the outcome of ART.[13,16]

Among the contributing factors to ROS in the ART setting, sperm centrifugation has a more profound deleterious effect on sperm function. Centrifugation of immature spermatozoa during semen preparation has been shown to generate ROS, producing adverse effects on sperm function, and consequently reducing fertilization potential.[17] For such reasons, innovative lab techniques are continuously devised and improved to maintain sperm quality and function. A means to properly measure oxidant and TAC levels may be indicative of the extent of OS present in a sample as well as improve the rate of successful fertilization during assisted reproduction.

Measuring Oxidative Stress

Sperm functional assays have been shown to be highly predictive of IVF outcome and potentially valuable in clinical decision-making.[18] Thus, the ability to accurately measure ROS in semen is essential in the assessment of OS and can provide tremendous value in monitoring and improving fertility outcome. This chapter provides an overview and methodological approach to some of the most common lab techniques currently used in the direct as well as indirect measurement of ROS in semen (Figure 13.2).

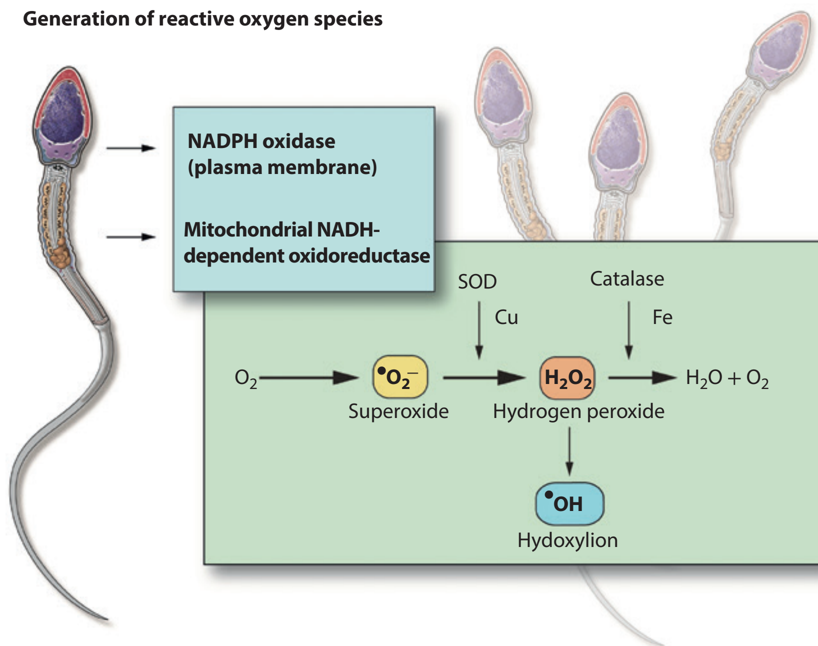

FIGURE 13.2 Generation of reactive oxygen species (ROS) via reduced nicotinamide adenine dinucleotide phosphate (NADPH) and NADH. Superoxide dismutase (SOD) and catalase assisting in the generation of ROS.

Methods to Measure Oxidative Stress

The following laboratory techniques/assays are most commonly used in the measurement of ROS in semen directly or indirectly (Figure 13.3):[19]

- Chemiluminescence assays
- Nitroblue tetrazolium (NBT) test
- Flow cytometry
- Immunohistochemistry
- Western blotting

Chemiluminescence

Chemiluminescence is the most well-established and widely used assay for measuring ROS in semen samples. In general, luminescent assays are very sensitive due to their ability to multiply and amplify a signal. Chemiluminescence takes advantage of an analyte's molecular properties. The principle behind the assay is for an enzyme to convert a substrate to a product, whereby photons of light are emitted as the reaction proceeds. A substrate is excited via oxidation and catalysis forming high-energy intermediates. The emitted light, or portrayed luminescence, represents the return of electrons from an excited to ground state. The instrument detects the luminescent signal and measures the emitted photons in relative light units (RLUs) that are typically proportional to the amount of ROS present in the sample.

Chemiluminescence has the ability to measure both intra- and extracellular ROS.[20] The two most commonly used probes are luminol (3-aminophthalic hydrazide) and lucigenin (N,N-dimethyl-9,9-biacridinium dinitrate). Often an enhancer is included with a substrate to protect the enzyme and allow the reaction to proceed for minutes without a substantial decay in light output.

Luminol is an uncharged, membrane-permeable versatile chemical that when mixed with an oxidizing agent exhibits chemiluminescence. It has the ability to react with different ROS (e.g., O_2^-, H_2O_2, and OH^-)

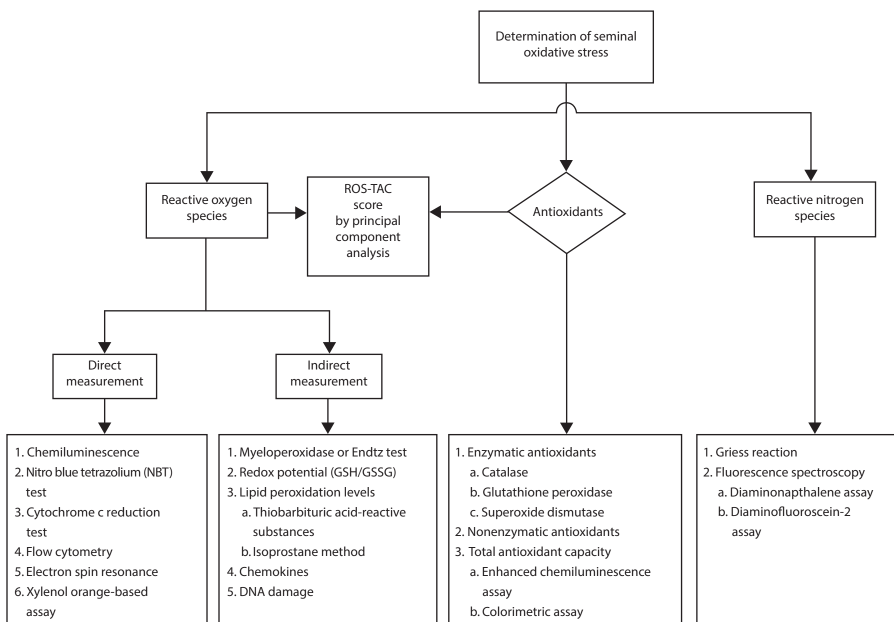

FIGURE 13.3 Flowchart showing various methods for measuring oxidative stress (OS). (From Lipshultz LI et al., *Infertility in the Male*, Cambridge University Press, Cambridge, UK, 2009. With permission.)

at a neutral pH, owing to its extreme sensitivity.[21] Luminol-dependent chemiluminescence can detect O_2^- and H_2O_2 due to the neutralizing effects of catalase and SOD, respectively. This has the ability to disrupt and diminish the detected luminescent signal. Reaction with free radicals causes single-electron oxidation of luminol and the generation of excited intermediates. As these high-energy intermediates fall to ground state, they emit energy that is detected as a light signal. A luminometer then converts this light signal into an electrical signal (photon with a wavelength of 425 nm), thereby quantifying the ROS in RLUs.

Lucigenin is another probe that can be used in chemiluminescence. It can react with a variety of reducing agents. For instance, it is especially sensitive in analyzing the enzymatic reaction producing H_2O_2 from O_2^- via SOD. The ability of SOD to enzymatically reduce O_2^- causes suppression of lucigenin-dependent cellular signals, and thus, provides a means to effectively measure O_2^-.[22] Mechanistically, a one-electron reduction activates lucigenin. This results in the formation of a cation radical-form of lucigenin that rapidly couples with O_2^- to yield dioxetane.[23] Dioxetane then decomposes into an excited N-methylacridone compound, which spontaneously emits blue light upon returning to its ground state.[21] The intensity of light emitted can be used to measure the amount of O_2^- present.

In comparison to lucigenin, luminol serves as a more advantageous probe for two potential reasons. First, lucigenin is specific to extracellular release of O_2^- only, whereas luminol can measure both intra- and extracellular ROS. Second, lucigenin is affected by various metal ions that may enhance or inhibit the intensity of the signal, whereas luminol is least affected by such factors.[24] Although luminol can quantify O_2^-, H_2O_2, and OH^-, it cannot distinguish between them, which is a limitation of this probe.[20] Nevertheless, luminol's high sensitivity makes it a preferred choice to measure global levels of ROS under physiological conditions.[20]

On the basis of the design of the luminometers, single and double tube as well as multitubes can be used in measurement of the ROS in several samples simultaneously (Figure 13.4). Table 13.2 gives an overview of some commercially available luminometers.

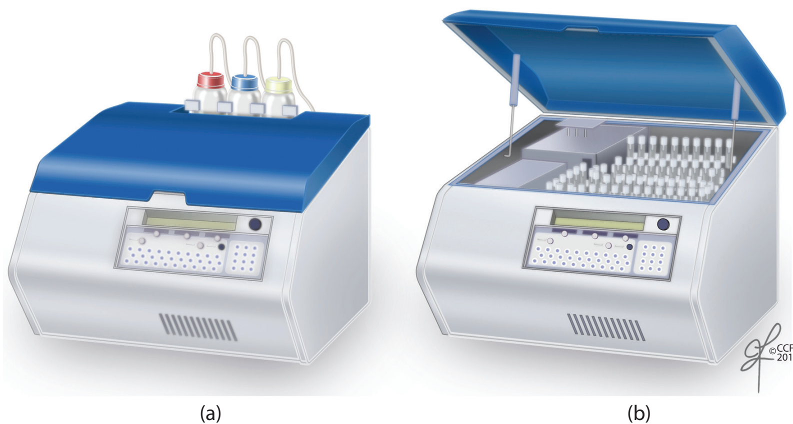

FIGURE 13.4 Autolumat 953 and luminometers used to measure ROS. (a) External view and (b) internal view. Multiple tubes can be loaded at a time. The luminometers can be connected to the computer to monitor all of the steps on the screen.

TABLE 13.2

Commercially Available Luminometers: Type, Sensitivity, and Manufacturer

Model	Type	Sensitivity and Dynamic Range	Manufacturer
GloMax 20/20	Single tube	0.1 g luciferase	Promega Cooperation
FB-12	Single tube	1000 molecules of luciferase	Zylux Cooperation
Triathler	Single tube	<10 amol adenosine triphosphate (ATP)/vial	Hidex
Optocomp-2	Multiple tube	0.1 pg ATP	MGM Instruments
Autolumat Auto Plus LB 953	Multiple tube	5 amol of ATP	Berthold Technologies

Measurement of ROS Using Chemiluminescence

Equipment and Materials

1. 15 mL disposable polystyrene tubes with caps
2. Pipettes
   a. Eppendorf: 5, 10, 50, and 1000 µL
   b. Serological: 1 and 2 mL
3. Centrifuge
4. MicroCell slides
5. Dimethyl sulfoxide (DMSO)
   a. Luminol or lucigenin
   b. Polystyrene round-bottom tubes
   c. Luminometer
   d. Phosphate-buffered saline (PBS) solution

Reagent Preparation

1. Stock luminol (100 mM): 177.09 g luminol should be added to 10 mL DMSO solution in a polystyrene tube. Due to luminol light sensitivity, the polystyrene tube must be covered in aluminum foil. This can be stored at room temperature until the expiration date.
2. Working luminol (5 mM): 20 µL luminol should be mixed with 380 µL DMSO in a polystyrene tube covered with an aluminum foil. Store the solution at room temperature.
3. DMSO solution: ready to use as is. Store at room temperature

Specimen Preparation

1. Once the semen sample has arrived, allow it to liquefy in the incubator at 37°C for 20 minutes.
2. Manual semen analysis is performed for concentration and motility.
   a. The following semen samples may be used for ROS measurement: neat or unprocessed, washed, and sample prepared by swim-up method or density gradient centrifugation.
3. Upon liquefaction, volume, pH, and color are recorded.

ROS Measurement by Luminometer

1. Luminometers should be set up in a dark room with the computer attached.
2. Label 11 Falcon tubes and add the proper reagents as indicated in Figure 13.5. To prevent contamination, change pipette tips in between each addition.
3. Vortex the tubes to mix the aliquot properly.
4. Place 11 labeled tubes in the luminometers in the following order (refer to Figure 13.5 for proper distribution):
   a. Blank: tubes 1–3
   b. Negative control: tubes 4–6
   c. Patient sample: tubes 7 and 8
   d. Positive control: tubes 9–11
5. After the loading is complete, operate the luminometers in accordance to the manufacturer's instructions.

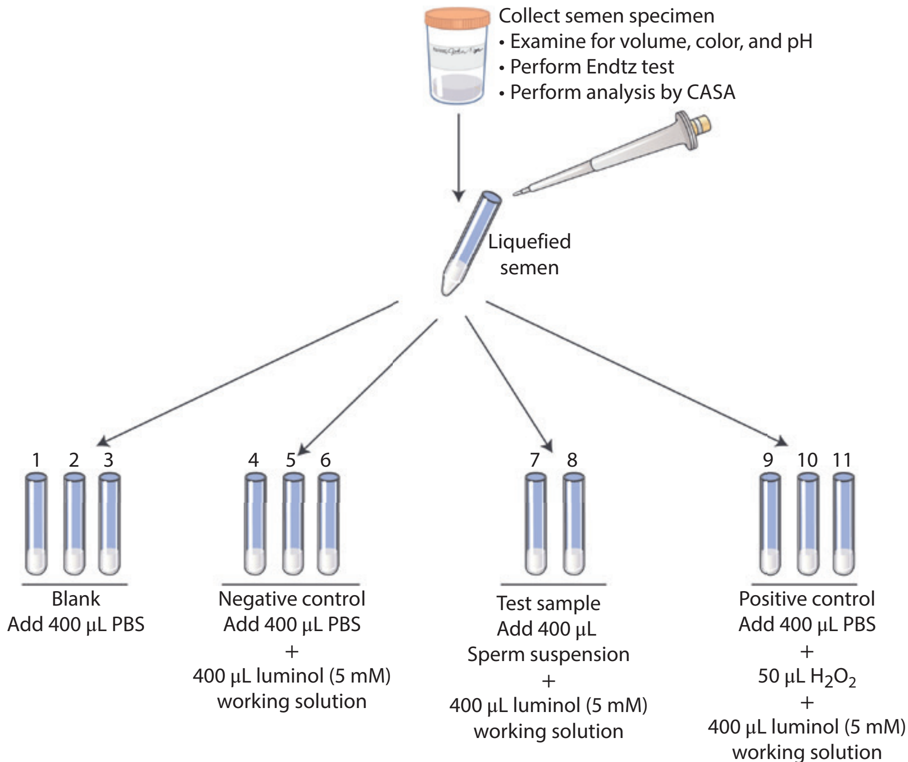

FIGURE 13.5 ROS measurement preparation. A total of 11 tubes are labeled from S1 to S12: Blank, negative control, patient sample, and positive control. Luminol is added to all tubes except the blank. H_2O_2 is added only to the positive control.

Calculating and Analyzing The Results

1. Calculate the "average RLU" for negative control, patient sample, and positive control.
2. Calculate sample ROS by subtracting the average from the negative control average: Sample ROS = Average "RLU mean" of sample – Average "RLU mean" of negative control.
3. Correct the sample for ROS by dividing it by "sperm concentration/mL."
4. Each lab should have established reference values for ROS concentrations.
   a. American Center for Reproductive Medicine Reference Values
      i. Normal range: <102.2 RLU(s)/$\times 10^6$ sperm
      ii. Critical values: >102.2 RLU(s)/$\times 10^6$ sperm

Precautionary Measures

Several factors such as reactant concentration, sample volume, reagent injection, temperature control, and background light can affect chemiluminescent reactions.[25] To obtain accurate and consistent results, the laboratory personnel operating the assay should take these elements into account. The reactant concentration can affect the amount of light that is luminated; thus, it is important to remain constant in this aspect. Time can play a vital role, as ROS measurement is most accurate and reliable when the sample is analyzed within the first hour of collection.[26] The chemiluminescence signal can also be altered by external sources of light. Phosphorescence has the potential to occur in almost any type of material, which will interfere with the assay.[27] A constant temperature between 22°C and 25°C should be maintained to ensure that the rate of the reaction does not increase.[27] In addition, viscous samples and poor liquefaction

have the potential to interfere with the emitted signals. Repeated centrifugation can cause an artificial increase in chemiluminescent signals due to the shear forces that are created from centrifugation.[26] The type of probe used can also affect the results, as luminol is sensitive to changes in the pH.[26]

Key Points

Although other options exist for monitoring ROS, chemiluminescence assay has continued to be the more commonly used and preferred choice. Its ability to accommodate more than 40 samples in one day allows for an efficient andrology laboratory, making it a very valuable tool for research scientists, technicians, and clinicians alike.[27]

Nitroblue Tetrazolium Test

Although chemiluminescence is the most well-established and popular method to measure total ROS, the lack of specificity and quality control remain obstacles. A more affordable, readily available, easy-to-perform, and highly specific technique is the semiquantitative microscopic NBT test.[28]

Conventionally, the NBT test is used to study neutrophil function and cellular oxidative metabolism. NBT is a yellow, water-soluble, nitrosubstituted aromatic tetrazolium compound. It is used to stain individual cells, such as spermatozoa and leukocytes. Cellular O_2^- reacts with NBT to form a formazan derivative, which is subsequently reduced to formazan via electron transfer from cytoplasmic reduced nicotinamide adenine dinucleotide phosphate (NADPH) to NBT.[29,30] The NBT formazan deposits can then be counted microscopically to measure the amount of NBT reduced. This number reflects the ROS generating cytoplasmic activity in cells. Therefore, the NBT test has been proposed as a means to assess the differential contribution of spermatozoa and leukocytes to ROS in semen.[31,32]

Measurement of ROS Using NBT Assay

Equipment and Reagents

1. 10 mL disposable polystyrene tubes.
2. Pipettes (Eppendorf and Serological).
3. Centrifuge.
4. PBS.
5. Potassium hydroxide.
6. DMSO.
7. 0.1% NBT working reagent.
8. Enzyme-linked immunosorbent assay (ELISA) plate.
9. Microplate reader.
10. Dilute the 0.01% NBT stock with PBS (1:10) to get a 0.1% NBT working reagent.

Semen samples should be collected by masturbation after 3–5 days of abstinence. The sample should be analyzed for sperm count, motility, and morphology according to the World Health Organization (WHO) guidelines and washed in PBS in preparation for ROS measurement by the NBT assay.

Add 1000 µL PBS (pH 7.4) in 200 µL semen and centrifuge at 300*g* for 5 minutes. Discard the supernatant and add a 1000 µL PBS under same conditions. Remove the supernatant and resuspend the pellet in 200 µL PBS and divide into two aliquots (100 µL each). Duplicate 100 µL samples are incubated with an equal volume of 0.1% NBT working reagent (1:10 diluted by PBS from 0.01% NBT stock) at 37°C for 45 minutes. The sperm cells are then washed with formazan in PBS twice by centrifuging the samples at 500*g* for 10 minutes to remove all residual NBT solution, leaving only a sperm pellet containing formazan. The intracellular formazan product should be solidified in 60 µL of 2 M KOH and DMSO to allow for quantification. After 5 minutes, dispense the reaction mixture into an ELISA plate. The sample can then be measured spectrophotometrically using a microplate reader at 655 nm.

To quantify the results, ROS production is expressed in micrograms of formazan per 10^7 sperm. This is derived from a standard curve of absorbance values for known amounts of formazan substrate. ROS levels can then be extrapolated from the formazan vs. absorbance standard plot.

Precautionary Measures

Although the potential value of NBT assay in assessing the differential contribution of spermatozoa and leukocytes to ROS seems promising, several limitations linger. The technique is semiquantitative and prone to observer bias. In addition, NBT can be reduced by many cellular reductases, affecting the identification of a true cellular origin of ROS.[33] The varying cellular content of oxidoreductases may also alter the rate of NBT reduction.[33] Moreover, the use of the NBT test in clinical laboratories is limited without established normal ranges. Additional studies are needed to define set values on which to base results, to allow for clinical application.

Key Points

The current lack of an inexpensive, easy-to-perform assay to assess sperm OS continues to impede optimal clinical care. Physicians are more reluctant to offer advice or empirical treatment without laboratory evidence supporting an oxidative pathology. Thus, further development and improvement of the NBT assay shows great potential for use in a clinical setting due to its ability to identify sperm with oxidative damage, along with its low cost and simplicity.

Flow Cytometry

Flow cytometry is a semiautomated assay, which studies the antigen profile cells. It utilizes the Scatchard principle of antigen–antibody binding and fluorochrome-based detection system.[34] Flow cytometry was initially used for the measurement of DNA content. However, it is now utilized to evaluate sperm count, viability, acrosomal reaction, mitochondrial membrane integrity or potential, and ROS.[35]

The most common probes used for semen analysis or measurement of specific ROS damage are (1) 2´,7´-Dichlorofluorescin diacetate (DCFH-DA), (2) Dihydrorhodamine 123 (DHR-123), and (3) Dihydroethidium (DHE).[36] DCFH-DA passively enters the cell and a nonfluorescent DCFH-DA is formed by way of cellular esterase and cleavage of the diacetate group.[37] In the presence of ROS, DCFH-DA is oxidized into dichlorofluorescein (DCF) and a green light is emitted.[38] Uncharged, nonfluorescent dye DHR-123, a derivative of rhodamine 123, also passively enters into cells and becomes oxidized by ROS, forming R123.[37] The mitochondria accumulate and localize the cationic green fluorescent dye R123.[39] DHR-123, commonly used to measure oxygen burst in leukocytes, has been shown to be more sensitive than DCFH-DA in oxidant detection.[38] DHE is a fluorescent two-electron reduction product of ethidium. When DHE is attacked by an ROS like O_2^-, DNA-sensitive fluorochromes are produced, generating a red fluorescence on excitation of around 510 nm.[38]

Key Points

There are a few advantages in using flow cytometry for semen analysis. Flow cytometry has the ability to measure multiple markers at a time and can accommodate patients with a low sperm count due to its small spermatozoa requirement.[40] In addition, it provides a means of high statistical power, thereby creating a rapid, reproducible, and accurate assay.[41] Relatively speaking, flow cytometry can be considered an expensive technique that requires technical expertise when compared to NBT assay. The routine use of flow cytometry for diagnostic purposes is not recommended at this time.

Oxidation–Reduction Potential

Despite the consistency and validity of methods such as chemiluminescence, flow cytometry, and the NBT test, there has always been a drive within the realm of andrology to find a more convenient and rapid way for measuring ROS in human spermatozoa. A new machine has recently emerged called

FIGURE 13.6 RedoxSYS system used to measure oxidation–reduction potential (ORP).

the RedoxSYS system. It measures an aqueous system's capacity to either release or accept electrons from chemical reactions ([i.e., oxidation–reduction potential [ORP]).[42] ORP, or redox potential, is the integrated measurement of total oxidants and reductants.

In short, a biological sample is applied to a RedoxSYS sensor and then inserted into a galvanostat-based reader. The test begins once the sample fills the reference electrode, completing the electrochemical circuit. The reader will then apply a small current sweep to the sample, causing complete exhaustion of important antioxidant species. This results in a calculation of the antioxidant capacity, which reflects the amount of electrons applied to the sample that causes the antioxidant depletion. This value is reported as capacity ORP (cORP) on the RedoxSYS system (Figure 13.6). cORP is the amount of antioxidant reserves available and relates to the ability of the system to respond to illness or injury.[43,44] The RedoxSYS system also gives a measurement of the static ORP (sORP), which is the tendency of a system to either donate or accept electrons. This value correlates with illness, severity of injury, and mortality. A higher sORP reading will indicate the presence of OS.

Measurement of ORP Using RedoxSYS System

The measurement of ORP using the RedoxSYS system requires the following items: pipettes (Pasteur, Eppendorf, and Serological), RedoxSYS analyzer, sensor, and calibration key.[44,45] Prior to ORP measurement, a routine semen analysis should be performed on the sample.

The sample used for ORP analysis can be either fresh or frozen semen or seminal plasma. A 20–40 µL sample should be applied to the RedoxSYS sensor. When the RedoxSYS system is ready, the "Insert Sensor" command appears on the display screen. RedoxSYS sensor should be inserted into the sensor socket. Proper execution of the test is indicated by the blinking of the blue testing LED light. Audible beeps will then signal the completion of the test with sORP and cORP appearing on the display screen.

Abstract Findings

The American Center for Reproductive Medicine at the Cleveland Clinic has recently assessed the effect of storage at subzero temperatures on the ORP of semen and seminal plasma samples. Findings indicated that the storage of semen and seminal plasma at –80°C did not have an effect on the sORP.[45] In addition, cORP also correlated well between the fresh and frozen samples.[45]

A prospective study was also conducted on the measurement of ORP in semen and seminal plasma samples, as well as a possible correlation between ORP and sperm motility. The study found that the RedoxSYS system accurately measured sORP and cORP in both semen and seminal plasma.[46] Furthermore, an sORP level of 4.73 $mV/10^6$ sperm in semen and 4.65 $mV/10^6$ sperm in seminal plasma was found to be a high predictor of abnormal sperm motility.[46] These results will be validated in future using a larger cohort.

Immunocytochemistry and Western Blotting

The ability of ROS to cause lipid membrane peroxidation and DNA damage has been well documented. However, studies examining their ability to alter protein function remain limited. Proteins play an instrumental role in practically all aspects of cellular life, acting as catalysts and processing signals internally and externally. Thus, the changes that ROS can induce may be harmful. Alterations in the activation or inhibition of transcription factors, signal transducers, and enzymes are all possible. The posttranslational modification (PTM) of proteins by ROS can be found in a number of pathological diseases, including infertility.[47] ROS induce three principal types of PTM of proteins: S-glutathionylation (GSS-R), nitrotyrosine (Nitro-Y), and carbonlyation. Through the principles of Western blot and immunocytochemistry, detecting protein PTM by ROS can create a biomarker and serve as another tool for evaluating infertility.

Western blot is a technique that is often used to separate and identify specific proteins based on molecular weight, isoelectric point, or electric charge via gel electrophoresis. The proteins are transferred to a nitrocellulose or polyvinylidene fluoride membrane and a band for each protein is produced and incubated with antibodies specific to the protein of interest.[48] The unbound antibody is washed off, revealing the bound antibody to the protein. The thickness of the band corresponds to the amount of protein present.[48] Selective studies have used Western blot to demonstrate that when sperm is exposed to OS-inducing conditions (e.g., cryopreservation or smoking), there may be a marked difference in sperm motility, viability, and acrosomal integrity due to protein degradation and phosphorylation.[49,50]

Immunocytochemistry is another method in biomedical research that can be used to identify proteins in tissues and cells. Similar to Western blot, immunocytochemistry makes use of antibodies that are capable of binding to proteins. The antibodies are typically linked to an enzyme or fluorescent dye, which gives off a signal that is detectable via microscopy. In spermatozoa treated with ROS, immunocytochemistry demonstrated tyrosine phosphorylation and alterations in motility and fertilization capacity.[51] With the potentially harmful effects of ROS on protein function and the ability of Western blot and immunocytochemistry to pick up on protein modifications, these techniques have a future value in translational medicine.

Conclusion

The effect of OS on human spermatozoa and its role in male infertility has been extensively studied. Yet, despite numerous reports and available assays, OS remains a major challenge in managing infertility. In most recent times, measurement of ROS by means of chemiluminescence has become a common laboratory technique. Newer techniques such as the RedoxSYS system have shown promise in providing a simple, fast, and accurate assay for measuring the oxidative potential in semen and seminal plasma. Further research is necessary to establish true cutoff values and place these techniques into greater clinical practice.

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The Importance of Sperm Surface Markers in Reproductive Success: Sperm Hyaluronan Binding

Leyla Sati and Gabor Huszar

Introduction

Our laboratory studied the development of the objective biochemical markers that would reflect the sperm maturation. We described the role of the sperm creatine kinase (CK) activity that reflects arrested sperm development at the level of cytoplasmic extrusion.[1–3] The next step in the sperm biomarker studies that we have identified contributing sperm fertility and function is the heat shock protein A2 (HspA2) chaperone protein, a component of the synaptonemal complex, and thus supports meiosis. The chaperone also supports cellular processes including DNA repair due to the role of HspA2 in transport.[4] We also demonstrated that low HspA2 levels are also associated with increased levels of DNA fragmentation. We assessed sperm chromatin maturity via aniline blue staining, which stains persistent histones in the sperm nucleus.[5–7] Increased levels of persistent histones in turn indicate a break in the developmental sequence of histones–transition proteins–protamines, which significantly affects DNA chain folding and vulnerability for increased DNA fragmentation. Finally, we have found that spermatozoa that are able to bind to solid-state hyaluronic acid (HA) are fully developed and are devoid of cytoplasmic retention, excess persistent histones, apoptotic processes, DNA fragmentation, and apoptotic marker of caspase-3, and show a normal frequency of chromosomal aneuploidies.[8–11] We discovered that HA binding is also related to sperm development and that the formation of the zona pellucida-binding and HA-binding sites is commonly regulated.

Structure of the Mature Spermatozoa

The events of spermiogenesis result in a structurally unique cell. Human sperm consists of a head and tail. The dense nucleus is covered by a vesicle called the acrosome.[12] It is bound to the nucleus by the perinuclear theca, which intervenes between the nuclear envelope and the inner acrosomal membrane.[13] The acrosomal cap that covers the anterior two-thirds of the nucleus contains enzymes such as hyaluronidase, neuraminidase, acid phosphatase, and a trypsin-like protease called acrosin. These acrosomal enzymes are crucial during the penetration of the zona pellucida of the oocyte.[14] The acrosomal enzymes are released when sperm touches the oocyte, which is the first step of the acrosome reaction. Sperm penetration and fertilization are facilitated by these complex processes and also prevent the polyspermy.

There is a minimum amount of cytoplasm in sperm cells. The sperm tail is subdivided into the neck, the middle piece, the principal piece, and the end piece. The short neck contains centrioles, microtubules, and mitochondria, helically wrapped around the coarse fibers and the axonemal complex. These structures are used for movement of the tail and fertilization. The principal piece contains the fibrous sheath external to the coarse fibers and the axonemal complex. The end piece is approximately 5 μm long and contains only the axonemal complex.

Human sperm shows variable morphology, making the clinical assessment difficult. Various sperm morphology classifications have been suggested since the early 1950s.[15,16] Sperm morphology is

expressed as a percentage of abnormal forms present in a sample. In recent years, the Kruger/Tygerberg strict criteria system has been most commonly used as a good predictor for male fertility.[17–20]

Spermatozoa enter the lumen of seminiferous tubules when fully formed. Then, they are pushed toward the epididymis by contractions in the wall of the seminiferous tubules. Although initially only slightly motile, spermatozoa obtain full motility in the epididymis. The activation of sperm is not entirely completed upon release from the male genital tract and is further modified while moving through the female reproductive tract. Once deposited inside the female reproductive tract, spermatozoa seek to reach the oocyte first and acquire hyperactivated progressive motility, defined as moving actively, either linearly or in a large circle, regardless of linear speed.[21]

Sperm Surface Markers

The quality of the epididymal maturation strongly influences sperm fertilizing capacity. Thus, sperm protein markers during maturation steps are among the most promising tools. However, only few sperm surface maturation proteins, such as fertilin, have been described with a role in fertility in large mammals because most studies were performed on rodents. Fertilin, a heterodimer complex composed of two integral membrane glycoproteins named alpha-fertilin (ADAM-1) and beta-fertilin (ADAM-2), as well as several other ADAMs, has been reported to be involved in sperm–oocyte recognition and in membrane fusion.[22,23] Fertilin binds to integrin α_6 β_1 leading to sperm–egg binding and membrane fusion.[24] The fertility of male mice lacking alpha-fertilin or beta-fertilin is substantially reduced due to sperm inability to migrate through the oviduct and to bind to the zona pellucida and to the oocyte plasma membrane.[22,25]

Although a highly predictive test for fertilization success remains to be developed, the potential use of HspA2 as a positive biomarker of fertilization success has been widely discussed.[26,27] Bromfield et al.[28] recently showed angiotensin-converting enzyme (ACE) and protein disulfide isomerase A6 (PDIA6) as potential HspA2-interacting proteins. The surface expression of PDIA6, but not of ACE, was shown to be dynamically regulated during sperm capacitation and, like that of previously characterized HspA2-interacting proteins, this surface expression proved vulnerable to oxidative stress.[28]

Regarding sperm–oocyte recognition, izumo sperm–egg fusion protein (IZUMO) and cysteine-rich secretory proteins (CRISPs) have previously been described in the literature.[29,30] Izumo-1 knockout male mice are sterile and sterility in these animals is primarily due to the failure of fusion of the sperm with the oolemma.[31] Izumo-1 binds to Juno protein expressed on egg oolemma and the interaction of Izumo-1 and Juno is conserved within mammals. The CRISP family of proteins is secreted at different sites in the epididymis and seminal vesicles.[32] It has been reported that specific types of CRISP remain firmly attached to the sperm surface even after in vitro fertilization (IVF) incubations.[33,34] Other remarkable sperm proteins indicated to have potential roles in fertilization are sperm lysosomal-like protein 1 (SLLP-1)[35] and a multifunctional thiol-disulfide oxidoreductase that can efficiently catalyze disulfide reduction, disulfide isomerization, and dithiol oxidation in substrate proteins, which is called ERp57.[36] Other studies have shown the relevance of CD9, a tetraspan membrane protein, on oolemma that is critical for the fusion of sperm membrane with oolemma by using CD9 knockout female mice.[37,38] Taken together, understanding of sperm-egg recognition mechanisms will definitely help us facilitate development of infertility treatment regimens and novel contraceptives. However, this picture is far from complete.

Hyaluronan Receptor Is a Marker of Sperm Development

Studies demonstrated that HA in the medium increased the velocity and retention of motility and viability in freshly ejaculated as well as in cryopreserved–thawed human spermatozoa.[39–41] HA effects on

sperm are likely to be receptor mediated as the presence of the HA receptor was detected in human sperm. Another important marker of normal sperm development is the sperm plasma membrane remodeling during spermiogenesis. Sperm plasma membrane remodeling is crucial because it facilitates the expression of the receptors for zona pellucida, along with those for HA (Figure 14.1).[11,40] HA in the extracellular matrix of the cumulus oophorus complex surrounding the oocyte can be used as a physiological selector of mature spermatozoa. Therefore, sperm that did not go through the remodeling process do not recognize zona pellucida or HA and fail in fertilization.

Various biochemical sperm markers have indicated thus far that the HA-bound spermatozoa exhibit nuclear, cytoplasmic, and shape properties identical to those bound to the zona pellucida of oocytes.[11,26,42] Indeed, a study of about 60 semen samples bound to both hemizonae and HA showed a significant correlation between the binding to the two entities at $r = 0.76$, $p < 0.001$.[11,26] We think that the correlation would even be closer if some of the hemizonae did not originate in unfertilized oocytes. The slides used in the sperm-HA binding tests are of uniform quality, with a very low (<5%) intra-assay variation in binding. We also demonstrated that the tyrosine phosphorylation patterns of sperm bound to either zona pellucida or HA were similar (Figure 14.2).[42] On the basis of the similarity in phosphorylation patterns of sperm bound either to zona pellucida or to HA, we proposed that there is a common regulatory pathway of tyrosine phosphorylation related to sperm ability. We believe that such ate regulatory pathway originated in the synchronous formation of the zona pellucida and HA receptors in the sperm plasma membrane following the remodeling process during spermiogenesis.

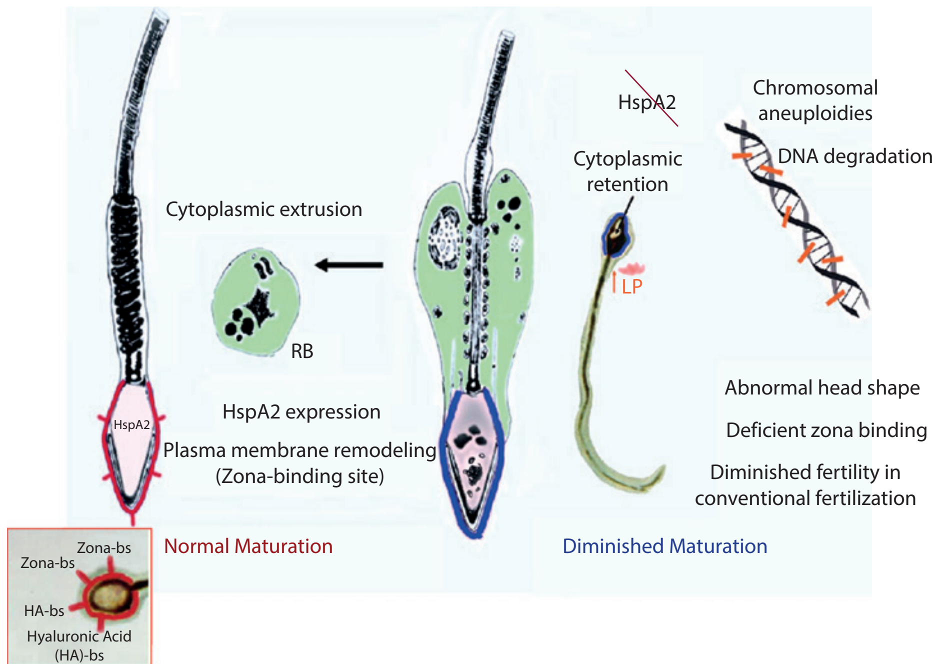

FIGURE 14.1 A model of normal and diminished/arrested maturation of human spermatozoa. During normal sperm maturation (left), elongating spermatids undergo cytoplasmic extrusion (represented by the loss of the residual body [RB]) and plasma membrane remodeling leading to the formation of the zona pellucida and hyaluronic acid (HA) binding sites (bs) (change from blue membrane to red membrane with the stubs). Spermatozoa of arrested maturity have low heat shock protein (HspA2) expression, increased levels of lipid peroxidation (LP), and consequent DNA fragmentation, abnormal sperm morphology, and deficiency in the zona and HA binding sites. (From Huszar G et al., *Reprod Biomed Online,* 14, 650–63, 2007. With permission.)

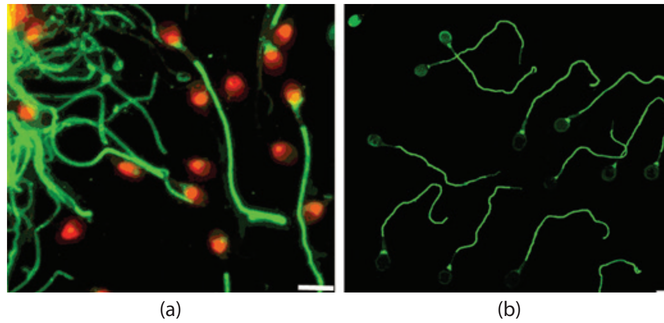

FIGURE 14.2 Tyrosine phosphorylation patterns in zona pellucida-bound sperm (a) and hyaluronic acid (HA)-bound sperm (b). Note that fluorescence is localized to the principal piece and the middle piece region of spermatozoa in an identical pattern within both the zona pellucida- and HA-bound sperm fractions. Bar: 10 µm. (From Sati L et al., *Reprod Sci*, 21, 573–81, 2014. With permission.)

Relationship between Sperm Shape and HA Binding

The potential relationship between sperm shape and genetic integrity has become very pertinent with the introduction of intracytoplasmic sperm injection (ICSI). ICSI sperm selection is not based on sperm–zona pellucida interaction as in conventional conception. In fact it depends on finding "the best-looking sperm" by eye or by nuclear features detected by specialized microscopy at IVF laboratories.[43] To test the validity of this concept, we first demonstrated that sperm retain their original shape after the steps of decondensation and denaturation.[44]

In line with these experiments, we found that numerical chromosomal aberrations can be present in sperm heads of any size or shape, but the risk is greater with amorphous sperm.[45] Even the most normal appearing sperm with normal head and tail size could be disomic or diploid, although diploidy is less prevalent with normal sperm dimensions and shape.[46] Thus, we concluded that sperm shape does not predict the presence or absence of chromosomal aneuploidies and sperm shape is an invalid parameter for selection of mature sperm without chromosomal aberrations for ICSI.[45] Considering the sperm analysis focused on sperm motility and concentration, the presence of the HA receptors during sperm development increases its prognostic value. In fact, we showed the enrichment of Tygerberg normal spermatozoa in HA-bound versus semen sperm fractions.[47]

HA Binding and DNA Integrity

The formation of mature spermatozoa requires a series of meiotic and mitotic changes in both the nuclear and the cytoplasmic compartments including the histone–transition protein–protamine replacement. First, somatic histones are replaced by testis-specific histone variants, which are then replaced by transition proteins in a process that involves extensive DNA rearrangement and remodeling.[48] During the final postmeiotic phases of spermatogenesis, sperm chromatin compaction occurs and almost 85% histones are replaced by protamines (protamine 1 and protamine 2).[49] In the end, sperm chromatin becomes a highly organized compact structure consisting of DNA and heterogeneous nucleoproteins.

We showed an association between diminished histone–transition protein–protamine exchange that may be detected by aniline blue staining of the excess persistent lysine-rich histones.[5] We designed the experiments in such a way that we double stained human spermatozoa, first with aniline blue and, after recording the sperm coordinates on the slide, applied a separate second probe for the same sperm.[7] Thus, we could provide evidence for relationships between the various biochemical markers of maturity/mismaturity. The biochemical attributes within the data pairs of aniline blue staining and Caspase 3 immunostaining (apoptotic process in the sperm) and aniline blue staining and DNA nick translation (DNA chain fragmentation/integrity studied in the same sperm) showed that there was a >70% agreement

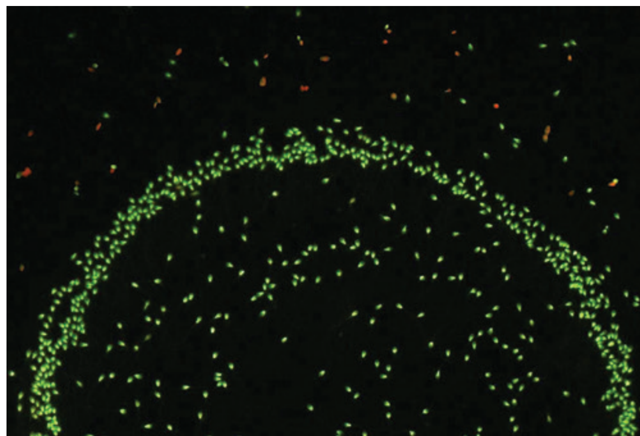

FIGURE 14.3 Acridine orange–stained sperm in the hyaluronic acid (HA) selection spot, and in the outside control area of the petri dish. Note the almost exclusive presence of sperm with green acridine orange fluorescence within the HA-selected sperm fraction. (From Yagci A et al., *J Androl*, 31, 566–72, 2010. With permission.)

between the patterns of marker pair staining within the same spermatozoa. The data indicated that the development/dysmaturity biochemical markers are related within the same sperm, and thus, the regulation of chromatin remodeling or the disturbed process of chromatin remodeling such as evolution from DNA–histone complexes to DNA–transition protein or DNA–protamine complexes is related to other attributes of sperm development or developmental arrest.[5]

We continued the chromatin maturity experiments with acridine orange staining probe.[50] This staining provides green fluorescence for DNA with high chain integrity and orange fluorescence for sperm with damaged DNA. It was reported that zona pellucida–bound sperm has mostly green fluorescence.[51] We performed this assay with sperm bound to the ICSI sperm selection device, the so-called PICSI dish (an IVF petri dish that carries an HA spot) (Origio Mid Atlantic, Mt. Laurel, New Jersey). Our results showed that virtually all of the HA-bound sperm exhibited green fluorescence (Figure 14.3).[50] Thus, whether probing sperm DNA with nick translation or with acridine orange, the DNA of HA-bound sperm showed high DNA integrity.

Clinical Importance of HA Binding

Further filtering effect of the zona pellucida has been reconstructed and tested by HA binding. No matter how high the aneuploidy frequency was in the semen sperm fraction, sperm bound and removed from HA had 4–6× lower disomy and diploidy frequencies within the 0.1%–0.2% normal range, which is customary in babies conceived with natural conception or with conventional IVF conception.[10] Thus, the PICSI dish seems to be an ideal platform for ICSI sperm selection (Figure 14.4).[10]

In the past few years, the sperm HA-binding assessment in the Andrology Laboratory and the PICSI dish has been increasingly accepted and used worldwide with excellent results in pregnancy rates and decline in early miscarriages.[52–54] In a recent study, Mokanszki et al.[51] examined the clinical success of ICSI with HA-selected sperm compared with conventional ICSI, as well as the necessity to differentiate patients according to the initial HA-binding assay (HBA) score and whether the sperm concentration or HBA score can provide additional information in 250 infertile couples (idiopathic infertile couples or infertility caused by male factor infertility).[54] The study reported significantly higher fertilization rate (FR) in the HA-coated PICSI group with >60% initial HBA, higher implantation rate (IR) in the PICSI group with ≤60% HBA, and higher clinical pregnancy rate (CPR) in every PICSI group compared with the ICSI groups ($p < 0.01$). A similar increase in IR and CPR, and lower PLR values was previously found by Worrilow et al.[52,53] Another study also showed that oocytes inseminated by HA sperm selection procedure had significantly higher FR in 50 couples undergoing ICSI using HA slides that they

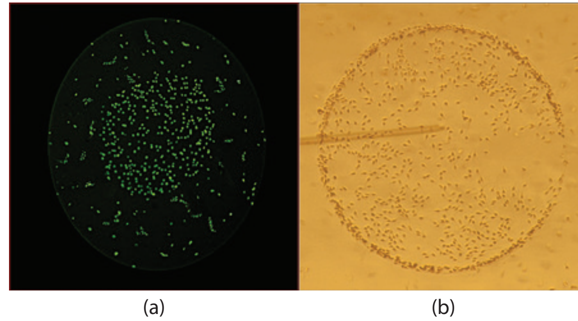

FIGURE 14.4 (a) Sperm approach from the periphery and then bind to the hyaluronic acid (HA) spot. (b) Sperm are picked up using the intracytoplasmic sperm injection (ICSI) pipette. (From Jakab A et al., *Fertil Steril*, 84, 1665–73, 2005. With permission.)

developed ($p < 0.05$).[55] However, the pregnancy rate and IR were insignificantly increased. Besides, a statistically significant correlation was reported between the sperm concentration and the HA-binding capacity ($p < 0.001$).[54] Therefore, the authors concluded that HBA screening prior to ICSI may be useful to increase clinical success.

Studies were also performed to compare conventional sperm selection and the use of sperm selected from a liquid source of HA. It was demonstrated that HA favors selection of spermatozoa without DNA fragmentation and with normal nucleus and an increased IR was reported.[56] It is important to note that no negative effect on the embryogenesis is reported using HA sperm selection for ICSI.

However, some other groups also published studies pointing out no association between HA binding and FR, fragmentation, and embryo quality though they used washed sperm.[57,58] Nijs et al.[59] investigated the clinical role of HBA in assisted reproduction in a prospective cohort study and a correlation of HA binding was reported with morphology, but it did not predict FR and CPR.[59] Another study indicated that there was no difference in the FR, number of top quality embryos, and CPR between the ICSI and PICSI groups in 156 patients. However, a higher PLR was observed in the ICSI group compared with the PICSI group but the difference was not statistically significant, suggesting that the clinical benefit from PICSI may be only when applied to a selective patient population.[60] Recently, Rashki Ghaleno et al.[61] evaluated the correlation of HBA with conventional semen parameters, lipid peroxidation, intracellular reactive oxygen species, DNA fragmentation, DNA maturity, and mitochondrial membrane potential level in human spermatozoa from 98 patients. The study also showed that HBA is sensitive to morphological integrity ($p < 0.01$), high progressive motility ($p < 0.05$), and nuclear maturation by means of DNA integrity ($p < 0.05$). However, no correlation with other conventional and intracellular sperm parameters was reported. In a recent Cochrane collaboration, the impact of HA sperm selection technique was evaluated on assisted reproductive technology (ART) outcomes.[62] Unfortunately, evidence was insufficient to allow review authors to determine whether sperm selected by HA binding improve live birth or pregnancy outcomes in ART, and no clear data on adverse effects were available.[62]

Conclusion

The data presented in this chapter support the validity of the HA-mediated sperm selection for ICSI by the HA-coated PICSI dish. The research base of the sperm plasma membrane remodeling during terminal spermiogenesis and the common origin of the formation of the receptors for zona pellucida and HA are novel ideas and are well supported by laboratory experiments to test its validity. The detailed characterization of HA-bound spermatozoa with respect to the lack of cytoplasmic retention, lack of persistent histones, lack of Tygerberg abnormal morphology, lack of apoptotic processes, and the close correlation of the rate of binding to zona pellucida or HA, all point to the high level of similarity between zona pellucida- and HA-selected spermatozoa.[8,10,11,47] The genetic properties of the HA-bound

sperm, with respect to the DNA integrity tested with the methods of either DNA nick translation or acridine orange fluorescence methods, and the data indicating the normal frequencies of chromosomal aneuploidies support the idea that the HA-selected spermatozoa are equivalent to those sperm bound to zona pellucida, no matter how high the rates were in the original semen sperm population. Thus, HA-mediated sperm selection provides an ICSI sperm selection method and initiates fertilization with the specially selected sperm that have no DNA fragmentation or chromatin abnormalities compared with those fertilizing sperm selected by the zona pellucida under physiological or conventional IVF conditions.[10,50,63]

Considering complete fertilization failure occurs in ~5%–15% of conventional IVF treatments,[64] defects in sperm-surface proteins and plasma membrane remodeling are vital in reproductive success. Thus, the fundamental understanding of the sperm surface markers will definitely help us improve IVF technologies and also provide new ideas about safe and prophylactic male contraception in the future.

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15

Importance of Sperm Surface Markers in Reproductive Success: Surface Charge

Luke Simon and Douglas T. Carrell

Introduction

The development of assisted reproductive technology (ART) procedures, particularly intracytoplasmic sperm injection (ICSI), enabled the use of any available sperm for assisted treatment.[1] Subsequently, sperm selection became an integrated process of ART, as the selection of healthier sperm for ART improved ART outcome.[2] Our understanding of sperm physiology, as well as the technology to select a healthier sperm, has progressively been improved, starting from sperm wash to more sophisticated separation techniques.[3] Although the external appearance of sperm has shown to be a poor indicator for sperm maturity[4] or its genetic qualities,[5,6] most of the methods currently available are based on sperm morphological features.[7] To test sperm quality for factors affecting ART outcomes such as DNA damage, histone retention, protamine content, and ratio, sperm must be either lysed or fixed, and the sperm tested for these parameters are unsuitable for use in ART. Therefore, new sperm selection methodologies to isolate healthier sperm without compromising its structural or functional integrity have been the goals for recent andrological research.[8,9]

It is well established that sperm selection is an essential part of all ART procedures. Currently, sperm selection for ART mainly relies on selecting motile and morphologically normal sperm, and techniques such as density gradient centrifugation (DGC) and swim-up are routinely been used for treatment. These methods improve the quality of selected sperm by selecting sperm with enhanced motility and removing nonmotile and dead sperm.[10] Despite the global use of these sperm selection approaches, it has become evident that sperm selection based on motility and morphology alone is inefficient to identify healthier sperm for fertilization.[11] In addition, the laboratory selection of sperm especially during ICSI has raised concerns about potential transmission of DNA alterations to next generations as a result of selecting suboptimal sperm for treatment.[5]

In all mammalian species, the ejaculated sperm are subjected to natural selection in the female reproductive tract to eliminate poor-quality sperm and enhance the chances of a successful reproduction.[12] These natural sperm selection processes are completely or partially absent during in vitro sperm selection process. In an attempt to select healthier sperm, several molecular features that mimic the natural sperm selection approaches such as hyaluronic acid binding, nonapoptotic sperm selection by annexin V labeling, and sperm surface charge[3,13–15] have been experimented. These new approaches have been associated with sperm function and are considered potentially important markers for selecting healthier sperm. Inclusion of these new molecular markers along with DGC has shown potential promise to enhance fertilizing ability and improve ART outcomes.[16–19]

Sperm Surface Charge as a Biomarker

All biological and nonbiological particles in nature are known to have an electrostatic potential. The surface of mammalian mature sperm is covered by a dense coating of carbohydrate-rich layer, which provides the sperm a net negative charge.[20] As a result of this negative charge, the sperm is showed to migrate toward the anode when placed in an electric field.[21] The sperm acquires its negative

charge during the maturation process within the epididymis.[22] This negative potential is not a fixed property of the sperm but rather a dynamic characteristic that is acquired by the sperm during its maturation process.[23] Modifications to the sperm membrane have been reported during maturation in the epididymis and during capacitation acrosome reaction in the female reproductive tract.[24,25] These modifications occur directly to the sperm membrane, a unique property of sperm that is distinct from somatic cells.

Sperm acquires motility and fertilizing capability through the process of maturation in the epididymis.[26] During its transit through the epididymis, many modifications to the sperm membrane occur as a result of interactions between the sperm membrane and the surrounding epididymal fluid.[27] The major components of epididymal epithelium secretions are glycoproteins that bind to the sperm membrane.[28] Among the glycoproteins, sialic acid residues are secreted as terminal sugars of sialo-glycoproteins that bind to the sperm membrane during the sperm epididymal transit.[29] The binding of sialo-glycoproteins to the sperm membrane results in a ~20- to 60-nm-thick glycocalys.[30]

The sperm glycocalys consists of 50–150 different carbohydrates residues linked to lipids and protein structures. Not all glycoproteins in the glycocalys are tightly attached to the sperm membrane. Some of the glycoproteins are anchored to the lipid-bilayer through glycosylphosphatidylinositol-anchored proteins, whereas others are superficially associated with the membrane via polar groups and hydrophobic interactions.[30] The glycocalys acts as an interphase between the sperm and the extracellular environment, whereas all interactions between the sperm and the environment or the oocyte should first initiate with the sperm glycocalys. Some authors have considered the presence of sperm glycocalys as a marker to measure sperm maturation.[27,31,32]

The acquisition of glycocalys is also associated with an increase in the negative surface charge during sperm maturation.[33,34] The negative charge is probably due to the acquisition of the negatively charged glycoprotein residues that make the glycocalys structure.[23] Some of the glycoproteins could be removed by washing the sperm,[30] which results in the removal of negative surface charge after sperm wash by DGC.[35] Removal of glycocalys by treating the sperm with neuraminidase is also shown to result in the loss of net surface negative charge.[36] The highly glycosylated sialic acid residues form a thick negative covering around the sperm, providing a net negative surface charge of –16 to –20 mV.[37,38]

Biological Importance of Sperm Negative Surface Charge

The concept of sperm maturation is primarily viewed as nuclear protein exchange,[39] nuclear compaction,[40] and membrane or surface modifications.[41,42] One of the most important modifications to the sperm membrane is the encapsulation of glycocalys over the sperm surface during the transit of sperm in the epididymis.[30] In mammalian species, CD52, a bipolar glycopeptide of epididymal origin, forms a major component of the sperm glycocalyx.[43] It is thought that the presence of high concentrations of sialic acid residue on the sperm membrane reflects normal spermatogenesis and sperm maturation. Compared with abnormal and immature sperm, mature sperm have higher net negative potential.[44]

Changes in membrane configuration during sperm maturation are important for various cellular interactions, such as cell-to-cell recognition, and sperm–egg interaction during fertilization.[45–48] The negative charge on the sperm surface also prevents the sperm from aggregation, nonspecific binding to the female reproductive tract and during storage.[43] Sperm with high negative membrane charge have been shown to be morphologically normal, contain high protamine content,[49] and have low levels of DNA damage.[3,49–51] Charge-separated sperm have been successfully used for assisted reproductive treatment[52] and resulted in a successful pregnancy following multiple ART failures using conventional sperm selection methods.[53]

Sialic acid residues not only provide the sperm its characteristic negative surface charge but also have a variety of biological functions in sperm. They are involved in masking of intrinsic protein antigens of the sperm membrane resulting in antirecognition molecules[54] and they also act as receptor determinants[55] by stimulating the production of naturally occurring antisperm antibodies. They also play a protective role to prevent premature loss of the acrosome content.[56] In mouse, the loss of sperm surface sialic acid results in phagocytosis of mouse sperm by macrophages in vitro.[29] In humans, seminal phagocytic cells play an important role in the elimination of abnormal sperm from the ejaculate.[57] In the

female reproductive tract, leucocytes are recruited at the human cervix in response to sperm.[58] However, the presence of surface sialic acid may prevent the sperm from phagocytosis.[33] Absence of sialic acid residues may lead to phagocytosis of sperm in the male and female reproductive tracts.

Charge-Based Sperm Selection Methods

Given a well-established role of glycocalys and its associated negative charge, only few research groups have utilized the charge-based selection to select healthier sperm. At present, three procedures of sperm separation have been described to utilize the sperm surface charge as its principle to select healthier sperm: Zeta test,[14] electrophoretic sperm separation,[59] and microelectrophoresis.[3]

Electrophoretic Sperm Separation

A research team led by Prof. John Aitken at the University of Newcastle in New South Wales, Australia, developed a novel technique of sperm selection known as electrophoresis sperm separation that utilizes net negative charge to select healthier sperm.[59] The presence of glycocalys coating on the sperm provides the sperm its net negative charge. Two models of electrophoretic systems have been developed: two-chambered and four-chambered devices. Under the influence of electric field, negatively charged sperm will be attracted toward the positive electrode. In the electrophoretic method, sperm is separated based on its size and charge. The four-chambered system consists of four separate compartments: two inner chambers (inoculation and collection) and two outer chambers. The inner compartments consist of an inoculation chamber (2 mL) into which semen is added and a collection chamber (400 µL) from which the selected sperm are collected. A polycarbonate separation membrane with pore size 5 µm and membrane area of 30 mm × 15 mm separates these chambers. The outer chambers are separated from the inner chambers by two polyacrylamide restriction membranes with a pore size of 15 kDa, which allows the movement of water and solutes between the inner and outer chambers, but traps the suspended cell particles within the inner chamber. The two-chambered system consists of an inoculation and collection chambers (Figure 15.1). The device hosts two platinum-coated titanium mesh electrodes and two 12V buffer pumps (one for each electrode chamber) running at 5 V, to circulate buffer through the chambers at a flow rate of 1.6 L/minute.

A detailed protocol for electrophoretic sperm separation is reported in Ainsworth et al. (2011).[60] Briefly, to isolate healthy sperm, 400 µL of semen and buffer (10 mm HEPES, 30 mm NaCl and 0.2 m sucrose;

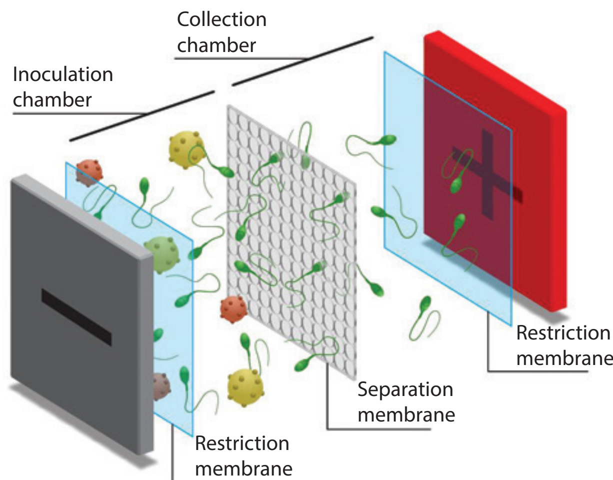

FIGURE 15.1 The two-chambered system consists of an inoculation chamber and a collection chamber.

pH 7.4 and 310 mOsm/L) were loaded into the inoculation and separation chambers, respectively, and allowed to equilibrate for 5 minutes prior to application of electric current. The samples were run at 23°C with a constant applied current of 75 mA and a variable voltage of between 18 and 21 V. When electric current is applied, the sperm with negative charge move from the inoculation chamber to the collection chamber through the polycarbon separation membrane. The 5-μm pore size of the membrane allows the passage of morphologically normal sperm, whereas larger cells such as immature germ cells, leukocytes, any contaminant, and large debris are left behind. The electrophoretic property of the sperm is explored in this method to isolate negatively charged sperm in large quantities from the collection chamber.[61]

Methodological Aspects of Electrophoretic Sperm Selection

The electrophoretic sperm preparation method is developed for its accuracy and speed of sperm recovery. It is well known that sperm are highly vulnerable to oxidative stress and have limited protection on their own.[62] The antioxidants in the seminal plasma protect the sperm from reactive free radicals generated from the leukocytes, contaminant cells, and the sperm itself.[63] When the sperm are removed from the seminal plasma, during the process of sperm preparation, the sperm become vulnerable to free radical mediated attack.[64] The prolonged centrifugation process itself is known to induce free radicals resulting in oxidative DNA damage.[65] In the SpermSep Cell Sorter 10 system, 2 mL of semen is loaded in the inoculation chamber, and after equilibration and 5 minutes of electrophoresis, 400 μL of sperm is obtained from the separation chamber. The rapid isolation of viable sperm without the centrifugation procedure prevents the sperm from oxidative-mediated DNA damage.[65]

A 5-μm polycarbonate membrane separates the inoculation and collection chambers. Semen is a heterogeneous mixture of various cell types (precursor germ cells, leukocyte subtypes, viable and nonviable sperm) and debris, which has to be removed during the process of sperm preparation. The membrane has an active area of 20 mm × 15 mm, which allows the passage of sperm but not contaminant cells. The inner chambers are secured by polyacrylamide restriction membranes that prevent cross-contamination between the semen and electrophoresis buffer while permitting free transit of electrolytes. The polyacrylamide restriction membrane keeps the system sterile and prevents sperm from cross-contamination. Overheating of the instrument during electrophoresis is prevented by maintaining the buffer at 25°C and circulating the excess buffer stored in the reservoir around the instrument using a pump.

Preferably, the electrophoretic system of sperm separation is rapid, free from contaminant cells, and able to isolate normal sperm with high percentage of morphologically normal and motile sperm with intact DNA.[59] The 400 μL of sperm obtained from the collection chamber could be directly used for intrauterine insemination procedures or for the purposes of in vitro fertilization and ICSI. The only drawback of the electrophoretic system is the laborious procedure of cleaning the instrument when compared with other sperm preparation methods. The components of the separation cartridge have to be autoclaved to ensure sterility. After each sperm separation, the electrophoresis buffer in the system is removed and the entire unit is rinsed with sterile distilled water. At the end of each day, the sterile distilled water is replaced by a cleaning buffer (0.1 M NaOH) and circulated in the electrophoresis unit for 30 seconds using the buffer pump. The cleaning buffer is left in the system overnight. The following day, the cleaning buffer is removed and the system is thoroughly rinsed out with at least three washes of sterile distilled water.[66]

Sperm Quality Following Electrophoretic Sperm Separation

1. *Recovery of sperm:* Ainsworth et al.[59] reported that the purity of the electrophoretically separated sperm preparations was extremely high, with virtually no contamination detected using phase-contrast microscopy. The recovery of sperm was also reported high; when 2 mL of semen with a mean sperm concentration ($52 \pm 5.2 \times 10^6$ mL^{-1}) was loaded into the inoculation chamber, after an initial 5-minute equilibration period 3.2% of sperm ($1.67 \pm 0.58 \times 10^6$ mL^{-1}) was recovered from the collection chamber without application of current. This is presumably due to sperm motility, where a population of motile sperm could pass through the polycarbon

separation membrane into the collection chamber. A 6.8% sperm recovery ($3.55 \pm 0.42 \times 10^6$ mL^{-1}) was observed after 30 seconds of electrophoresis, whereas 42.9% sperm recovery ($22.31 \pm 5.85 \times 10^6$ mL^{-1}) was observed following 15-minute electrophoresis.[59] The electrophoretic system was also efficient to isolate testicular sperm exhibiting more residual motility, with no contaminant cells being detected in the collection chamber following the use of testicular biopsy material.[52]

2. *Sperm motility:* The percentage of sperm motility was comparable between the original semen and the sperm separated by electrophoresis. These percentages are also maintained when sperm was obtained at different electrophoretic duration. These experiments suggest that the negative charge of the sperm is not the only factor that is potentially involved in the electrophoretic isolation of sperm. It is possible that the motility of the sperm also may contribute to the isolation process, where the sperm could swim through the pores to the collection chamber. When sperm were immobilized by exposing to benzoquinone and introduced in the electrophoretic system, a significant reduction in the recovery of sperm was observed in the absence of electric field. However, when electrophoresis was performed, no significant change in sperm recovery was observed, although sperm recovery levels were reduced.[60] This experiment proves that the sperm selected from the electrophoretic system involves a combination of both sperm motility and cell surface negative charge for the isolation of high-quality sperm.

Interestingly, at higher electrophoretic field settings a progressive loss of total sperm motility was observed.[65] Analysis of sperm kinematic characters of the sperm by computer-assisted sperm analysis CASA system showed that the quality of the sperm motility did not significantly change between the original semen and isolated sperm regardless of the duration of electrophoretic separation.[59] When cryopreserved semen was used in the electrophoretic system, the sperm population isolated after 5 minutes of electrophoresis showed significantly improved motility compared with the residual semen counterpart.[52] In the presence of highly immature sperm found in the testicular biopsy, the electrophoretic system was able to isolate a subpopulation of sperm that exhibited slight motility compared with the unselected population.[52]

3. *Sperm vitality:* The percentage of viable sperm isolated after electrophoretic separation was consistent with the values of the original ejaculate.[59] In addition, the vitality of the sperm did not change with the duration of electrophoretic time and at different electrophoretic power settings. However, when electrophoresis was performed on cryopreserved semen, the sperm population isolated after electrophoresis contained significantly higher viable sperm.[52] Similarly, an increase in viability was observed in the subpopulation of immature sperm electrophoretically separated from testicular biopsy.[52]
4. *Sperm morphology:* The percentage of sperm with normal morphology was increased following electrophoretic separation. The percentage of morphologically normal sperm in the electrophoretically separated sperm was higher regardless of the duration of electrophoresis, and with no significant variation between different electrophoresis time periods.[59] Sperm deformity index is another expression for sperm morphology and is a known predictor of fertilization in vitro.[67] The values of sperm deformity index at any point for electrophoretically separated sperm were significantly lower than 0.93 (a threshold value for sperm deformity index to determine in vitro fertilization[68]).
5. *Sperm DNA fragmentation:* A significant reduction in the level of sperm DNA damage was observed in the sperm separated using electrophoretic system when analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. This significant reduction in DNA damage was observed for all time points up until 10 minutes of electrophoresis time, after which no statistical change in the percentage of DNA-damaged sperm was seen.[59] In an ejaculate, the DNA fragmentation index (DFI) measured by sperm chromatin structure assay (SCSA) was also reduced following electrophoretic sperm separation from 41% to 15% DFI.[52] Reduction in sperm DNA damage following electrophoretically sperm separation was observed after the use of cryopreserved and testicular biopsy samples.[52]

6. *Genotyping of electrophoretically isolated sperm:* Ainsworth et al.[60] performed a definitive PCR-based technique to determine the ratio of X- and Y-bearing sperm separated by the electrophoretic system, based upon the structure of the amelogenin gene, where a single oligonucleotide primer set was used to amplify two easily distinguishable DNA products from the X and Y chromosomes of 977 bp and 788 bp, respectively. The amplicons of the X chromosomes bearing sperm are 189 bp larger than the amplicons of the sperm bearing Y chromosome. When sperm selected from electrophoretic separation were analyzed using this PCR strategy, there was no significant deviation from the anticipated 1:1 ratio of X- and Y-bearing sperm. Therefore, the use of sperm selected from the electrophoretic systems in ART should have no impact on the gender of the resultant offspring.

Comparison between Electrophoretic Sperm Separation and Other Methods

The recovery of sperm was identical between electrophoretic sperm selection with Percoll centrifugation and DGC.[59,61] Sperm motility was higher after Percoll gradient centrifugation and DGC when compared with electrophoretic separation, but all methods enhanced the percentage of viable sperm compared with raw semen. However, the percentage of sperm with normal morphology was higher, whereas sperm deformity index and the percentage of sperm exhibiting oxidative DNA damage (8-hydroxydeoxyguanosine) were lower after electrophoretic separation compared with Percoll.[65] The level of sperm DNA damage was lower only in the case of electrophoretically isolated sperm when compared with Percoll gradient and raw semen.[59] The separation duration for preparing a single sample is just 5–10 minutes electrophoresis, which is considerably shorter than the 20 minutes required for sperm preparation by DGC.

Clinical Importance of Sperm Preparation by Electrophoretic

Ainsworth et al.[52] reported the first successful clinical pregnancy following ICSI using sperm separated by the electrophoresis method. Since then, there has been no large prospective controlled trial to prove the suitability of electrophoretically separated sperm in clinical settings. Fleming et al.[61] reported a small prospective controlled clinical trial, involving 28 couples, 17 of which were undergoing IVF and 11 ICSI treatments. This was performed as a split-sample cohort study design, where sperm were prepared by both DGC and electrophoretic separation. In this study, there was no significant difference in fertilization rates (63.6% vs. 62.4%), embryo cleavage rates (88.5% vs. 99.0%), or the percentage of top-quality embryos (26.1% vs. 27.4%) obtained following the insemination of sperm preparation by DGC and electrophoretic separation, respectively.[61] The lack of statistical significance in fertilization rate and embryo quality was observed in both IVF and ICSI patient groups. Given the small number of patients enrolled in each group, the study reported two ongoing pregnancies after the transfer of 13 DGC-derived embryos compared with five ongoing pregnancies after the transfer of 23 embryos derived from electrophoretically separated sperm. Although these numbers are not sufficient to obtain a statistical difference, this study provides the proof-of-principle, that electrophoretically separated sperm could be used for ART.

In recent years, sperm banking or cryopreservation has been reported not only in patients referred for ART but also in men undergoing treatments such as vasectomy or cancer. To confirm the clinical use of the electrophoretic system in these patient groups, sperm selected from cryopreserved semen and testicular biopsies were subjected to vitality, motility, morphology, and DNA damage analysis.[52] Five-minute electrophoretic separation generated 27% sperm recovery from cryopreserved semen and 28.4% sperm recovery from testicular biopsies. In both the conditions (cryopreserved semen or testicular biopsies), the recovered subpopulation was enriched in vitality and morphologically normal sperm. The enhanced vitality and morphological normal sperm, and a reduction in the levels of DNA damage prove that the methodology is sufficient to select healthy sperm for ART.

Conclusion

In principle, the electrophoretic sperm separation procedure has great potential as an extremely versatile and cost-effective method to prepare sperm. The sperm isolated by this method are reported to have good

recovery rate, an improvement in vitality and sperm morphology, and a reduction is sperm with DNA damage. Therefore, sperm selection by this process appears to offer more promise as a fast, efficient method for isolating sperm suitable for ART treatments. However, additional studies are required to confirm the effectiveness of this electrophoretic method in the management of male infertility and as a technique to improve ART success.

Zeta Test

Ishijima et al.[37] were the first research group to illustrate Zeta potential, which was defined as the electrostatic potential between the sperm membrane and the surrounding medium. The negative electrical charge of the sperm surface is termed Zeta potential or electrokinetic potential. Thus, sperm selection based on their surface charges was established as Zeta potential. Later, Chan et al.[14] developed the Zeta test to select sperm according to the electrical potential of the sperm.

Methodology of Zeta Test

Zeta test should be carried out immediately following DGC, as sperm cells become less negatively charged with the onset of capacitation. To isolate highly negative charged sperm, it is essential to use a new (untouched) centrifuge tube (15 mL) the electrostatic charge of new tubes is high. DGC washed sperm (0.1 mL) is diluted with 5 mL of serum-free HEPES–HTF medium and gently pipetted in the tube. Extreme care should be taken to place the tube inside a latex glove up to the cap and hold the cap of the tube at all time. The tube carries a positive charge and grounding of the tube would reduce the level of positive charge and binding of negatively charged sperm. The tube with the sperm sample should be rotated two or three turns clockwise and incubated at room temperature (23°C) for 1 minute to allow adherence of the charged sperm to the wall of the centrifuge tube. Following incubation, the tube is centrifuged at 200 × *g* for 5 minutes and the tube is slowly inverted to drain out all nonadhering sperm and other contaminant cells. The excess liquid at the mouth of the tube is removed by placing the tube upside down on a tissue paper. Three percent serum supplemented with HEPES–HTF medium (0.2 mL) is pipetted into the tube, allowing the medium to trickle down the side of the tube wall. This process helps neutralize the positive charge of the tube and detach the adhering sperm from the wall (Figure 15.2). The collected medium at the bottom of the tube is repipetted and used to rinse the wall of the same tube several times to increase the concentration of recovered sperm.[14,51,69]

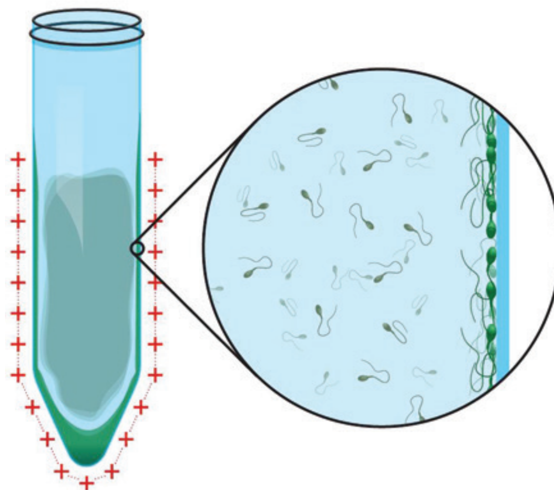

FIGURE 15.2 A 3% serum supplemented with HEPES–HTF medium (0.2 mL) is pipetted into the tube, allowing the medium to trickle down the side of the tube wall. This process helps to neutralize the positive charge of the tube and detach the adhering sperm from the wall.

Quality of Sperm Separated by Zeta Test

Chan et al. (2006)[14] showed that the Zeta test is a simple and reliable method to select mature sperm. The electrostatic potential of healthier sperm in an ejaculate is shown to be mostly electronegative.[44,43] A recent study proved that sperm selected based on its Zeta test are more mature when assessed for markers such as protamine content, ability to resist DNA fragmentation, or apoptotic markers such as TUNEL or acridine orange.[16] The Zeta test has been shown to isolate sperm with significantly higher normal morphology, hyperactivation, DNA integrity, and maturity, when compared with control samples; however, the process of sperm binding to the surface charge of container reduces its motility.[14,16,17,49,51,69] Another advantage of the Zeta test is that its potential is unaltered after cryopreserved-thawing process.[69]

Comparison between the Zeta Test and Other Methods

The percentage of DNA fragmented was significantly reduced in sperm isolated from Zeta test compared with DGC.[16,51] The efficiency of the Zeta test to isolate sperm with normal DNA integrity was 62% while that of DGC procedure was 46% when compares to control group.[51] However, when Zeta and DGC selected sperm were compared for CMA3 positivity, there was no significant difference between the two methods.[16] The efficiency of the Zeta test to separate sperm with normal protamine and intact DNA appears to be higher than that of the DGC procedure, when analyzed using four sperm integrity parameters CMA3, TUNEL, sperm chromatin dispersion assay and acridine orange test.[16] When Zeta test was compared with hyaluronic acid (HA) binding assay, the percentage of sperm normal morphology and CMA3 positivity were not significantly different between the two groups.[49] However, the percentage of DNA fragmented spermatozoa was reduced significantly in the Zeta group as compared with the HA group. The efficiency of the HA procedure relative to semen for normal morphology, DNA integrity and protamine content were 95%, 5.9%, and 19.1%, respectively, while those for the Zeta procedure were 67%, 44.6%, and 13.1%, respectively.[49]

Clinical Importance of Sperm Preparation by Zeta Test

In a study by Kheirollahi-Kouhestani et al. (2009),[16] sibling oocytes from 30 patients were split into two groups and inseminated by sperm prepared by DGC and by DGC/Zeta. The treatment groups were compared with the control group ($n = 34$) who underwent ICSI treatment during the same time period. Fertilization rate was significantly higher in sibling oocytes group inseminated by sperm prepared by DGC/Zeta compared with the DGC group (52.39 vs. 65.79%, $p = 0.032$). Fertilization rate was not different between the oocytes inseminated by DGC and the control group. Cleavage rate and embryo score on day 2 were not significantly different between DGC and DGC/Zeta insemination groups. Embryo score on day 3 after DGC/Zeta insemination was higher when compared with the control group, but was not statistically significant ($p = 0.09$). The pregnancy and implantation rates in couples receiving at least one embryo from the Zeta group ($n = 28$) were 53.57% and 26.18%, respectively, whereas in the control group ($n = 34$), the pregnancy and implantation rates were 33.33% and 15.80%, respectively, but these improvements were not statistically significant. The results suggest improved pregnancy and implantation rates in individuals who received embryos from the DGC/Zeta group compared with the control group; however, the improvement was not statistically significant. In a case study by Deemeh et al. (2010),[53] 10 oocytes inseminated by sperm selected from DGC/Zeta method resulted in 90% fertilization rate and eight good embryo quality embryos. A successful pregnancy was obtained following transfer of three embryos on day 3.

Conclusion

The Zeta test is inexpensive, easy to perform, and does not require any complex equipment to select healthier sperm. However, low sperm recovery rate is a limitation for patients with oligozoospermia. In addition, this method of sperm selection may not be applicable for testicular or caput epididymal sperm[14] as they lack sufficient net electrical charge on the sperm membrane.[70]

Micro-Electrophoresis Sperm Selection Method

Scientists at the University of Utah, under the leadership of Professor Douglas Carrell, developed a novel method of sperm selection called "microelectrophoresis," based on the electrostatic properties of sperm.[3] Sperm surface charge is utilized in this method to select mature and healthier sperm. Sperm entering the epididymis are positively charged. During epididymal sperm maturation, sperm acquire negative charges, by binding of negatively charged glycoproteins to their membranes.[71] The research group found that the negative surface charge of sperm could be completely removed by vigorous sperm washing, resulting in positively charged sperm. They also found that accumulation of negative charges corresponds to the duration of time spent in the epididymis, and the epididymal sperm population displays a variable level of sperm surface charge ranging from high to low negative charge to positively charged sperm.[35] Whereas immature sperm have a low negative charge, older sperm undergo membrane phosphorylation, capacitation, and apoptosis, thereby losing their membrane integrity and surface glycocalys, resulting in loss of negative surface charge. Using the slight variation in the surface negative charge, the researchers devised a new method of sperm selection known as microelectrophoresis, through which it is now possible to visually identify sperm with slight charge differences and separate those possessing a highly negative charge.[3]

Methodological Aspects of Micro-Electrophoresis

The microelectrophoresis sperm separation unit consists of three parts[3] (1) the power supply, (2) the connecting electrodes, and (3) the disposable sterile electrophoresis unit (Figure 15.3). The power supply consists of a basic power-pack unit that can control and supply 0–300 V and 0–300 mA of electricity (Bio-Rad Laboratories, Inc.). Reusable platinum electrodes are used to connect the electrophoresis unit to the power supply.

The basic methodology for microelectrophoresis is essentially the same as for the ICSI sperm selection procedure. The microelectrophoresis apparatus was set up on the ICSI stage of an inverted microscope. Sperm were viewed at 200× magnification and picked up with a beveled, glass ICSI pipette connected to a CellTram Vario manual microinjector (Eppendorf). Two milliliters of electrophoresis buffer (10 mM Tris, 20 mM NaOH, pH: 7.8) was added to the electrophoresis chamber. Approximately 10–15 μL sperm were added to the electrophoretic buffer and allowed to settle for 2 minutes. Electrophoresis is performed by applying current between 6 and 14 mA (increased from low to high) at variable 30–100 V. Sperm were collected during electrophoresis (starting as soon as the electrophoresis was initiated, and finishing before it was completed). During electrophoresis, the sperm were viewed through the ICSI inverted microscope. The charge of the sperm was observed by visualizing the direction of sperm movement under the influence of current (PCS move toward the cathode and NCS move toward the anode). Similarly charged sperm (PCS, NCS, or neutral) were collected into the ICSI pipette and placed on a marked glass slide for further experimental analysis.

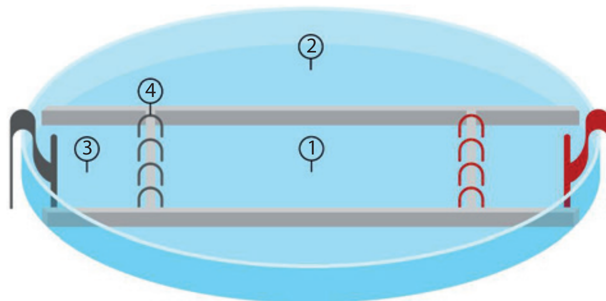

FIGURE 15.3 The micro-electrophoresis sperm separation unit.

In patients where the number of charged sperm was low, sperm were identified within the electrophoretic chamber at different microscopic fields of view by moving the ICSI stage. The sperm were separated at low current first (6 mA) to ensure the isolation of sperm with a high negative charge. The current was gradually increased to facilitate the movement of sperm in the electrophoretic field. During electrophoresis, sperm were assessed for their morphology, and sperm with normal morphology and negative charge were selected using the ICSI pipette. Fifty charged sperm were collected within 3–5 minutes of electrophoretic exposure. The electrophoretic duration varied between patient samples and according to the availability of charged sperm. The ratio of charged sperm in the native semen and prepared sperm was examined at 200–400× magnification: 200 sperm were evaluated per sample based on their movement in the electric field and classified into PCS, NCS, and neutrally charged sperm.[35]

Quality of Sperm Separated by Microelectrophoresis

In an experiment, 50 sperm were selected based on their charge (positive, negative and neutral) using an ICSI pipette ($n = 17$ samples), and compared with their control. Sperm DNA damage was higher in control (17.3% ± 3.2%, $p < 0.001$), neutrally charged (12.1% ± 2.1%, $p < 0.001$) and PCS populations (27.8% ± 6.0%, $p < 0.001$) when compared with the NCS (3.9% ± 1.5%) population.[3] In another experiment, negatively charged sperm were selected at low electric field (6, 8, and 10 mA) and compared with positively charged sperm and unselected control.[35] The results showed no differences in the level of sperm DNA damage when sperm were picked at lower electrophoretic current settings; however, at all current settings negatively charged sperm were shown to have significantly lower DNA damage, when compared with positively charged sperm and their corresponding unselected control.[35]

In both these experiments, sperm selected by microelectrophoresis showed relatively low DNA damage in the negatively charged population compared with unselected controls. In addition, the negatively charged sperm displayed significantly reduced DNA damage compared with the positively charged sperm. A sperm becomes positively charged when it completely loses the negatively charged CD52 glycoprotein anchored on its membrane. It is well known that apoptosis is a mechanism to eliminate dysfunctional cells.[72] During apoptosis in sperm, the integrity of the membrane is lost due to translocation of phosphatidylserine,[73] during which the phosphatidyl serine is shifted from the interior to the exterior of the plasma membrane.[74,75] In addition, apoptosis results in DNA fragmentation and degradation of cytoskeletal and nuclear proteins (reviewed by Elmore, 2007),[76] and affects fluidity of the plasma membrane.[77] The externalization of phosphatidyl serine and loss of membrane fluidity may also facilitate the loss of epididymal glycoproteins anchored to the membrane, thereby causing a loss in negative charge. In accordance with this hypothesis, selection of sperm with high negative charge may aid as a noninvasive biomarker to select nonapoptotic sperm.

Diversity of Charged Sperm in Raw Semen and DGC Prepared Population

When the charged sperm population was analyzed in the native semen,[35] an average of 94.2% ± 1.0% of the sperm displayed a negative charge. The percentage of charged sperm varied between individuals. Under the electric field, some sperm did not move toward cathode or anode; such sperm were considered neutral or with very low +/– charge.[35] By increasing the current during electrophoresis it was possible to move the low charged sperm toward the cathode or anode. At 14 mA, 1.6% ± 0.7% of the sperm were immobile (considered as neutrally charged) in the electric field.

The percentage of negatively charged sperm ($R^2 = 0.999$; $p < 0.001$) and positively charged ($R^2 = 0.998$; $p < 0.001$) increased with an increase in current. In contrast, the percentage of neutrally charged sperm decreased with an increase in current ($R^2 = -0.999$; $p < 0.001$). A similar trend was also observed in the negative ($R^2 = 0.998$; $p < 0.001$), positive ($R^2 = 0.983$; $p < 0.001$), and neutral ($R^2 = -0.993$; $p < 0.001$) charged sperm population after DGC.[35] After DGC, there was a decrease in the percentage of NCS and an increase in the percentage of positive and neutrally charged sperm. As native semen samples contained a significantly higher percentage of negatively charged sperm than DGC samples, it is likely that the process of sperm preparation by DGC removes the charged particles adhered to the surface of the sperm membrane.

Simon et al.[35] reported that about 60% of the sperm in the ejaculate possess negative membrane charge when observed under a low electrophoretic current setting of 6 mA. However, when the current is increased to 14 mA, 94% of the sperm show electrophoretic mobility toward the anode. A gradual increase in the population of NCS was observed as current was increased. These findings suggest that not all sperm in the ejaculate have identical membrane charge. They observed that less than 4% of the sperm in the ejaculate carry a positive charge, which can be identified by the movement of these sperm toward the cathode. The uptake of the epididymal secretions reflects the maturational status of the epididymal sperm.[41,43,78] In agreement with the published literature, their results support the notion that sperm membrane charge is a variable factor, which may depend on the accumulation of negatively charged glycoproteins to the sperm membrane.

It is well documented that some epididymal proteins are weakly associated with the sperm membrane,[79,80] whereas others are anchored to the membrane by glycosylphosphatidylinositol subunits (reviewed by Leahy and Gadella, 2011).[43,81] In human sperm, CD52, a lipid-anchored glycoprotein, provides the characteristic negative charge to the membrane.[30] Modifications in membrane configuration could disrupt the association between epididymal proteins and the sperm membrane. During cryopreservation (reviewed by Leahy and Gadella[81]) and early capacitation,[82] modification and redistribution of molecules to the sperm membrane could also cause the loss of the epididymal proteins from the sperm surface,[83,84] resulting in the loss of electronegativity.

Simon et al. [35] reported that the process of DGC appears to physically wash off the negative charge from the sperm. As a result of DGC, there is an increase in the percentage of positively charged sperm and a decrease in the percentage of negatively charged sperm. Interestingly, not all sperm in the ejaculate lose their negative charge. Although the process of DGC should have identical effect on all sperm, presumably the cells with a higher starting negative charge retain some of their negative charge, whereas the cells with low negative charge completely lose their charge to become positively charged sperm. This concept is supported by the observation that sperm with high negative membrane charge could be electrophoretically moved at low current (6 mA), whereas the immovable (neutrally charged) sperm could be moved toward cathode or anode by increasing the electrophoretic current. It may be that sperm with anchored CD52 glycoprotein[43] may retain the negative charge, whereas the epididymal proteins that are weakly associated with the sperm membrane are simply washed off the sperm surface. These results are in accordance with the published literature[43,79–81] suggesting that the epididymal proteins adhered to the sperm surface are removable.

Characteristics of Charged Sperm Population Analyzed by Microelectrophoresis

1. *Association of sperm charge with histone retention:* The percentage of sperm with normal histone retention was directly proportional to the percentage of negatively charged sperm and inversely proportional to the percentage of positively charged sperm. Although the percentage of sperm with abnormally high histone retention was inversely proportional to the percentage of negatively charged sperm and directly proportional to the percentage of positively charged sperm.[3] In another experiment, Simon et al. [35] confirmed the association between histone retention and charged sperm. In addition, they showed that the relationship grew stronger with increasing current. But when they excluded neutrally charged sperm, the relationship was identical at all electrophoretic current settings.

   Previous studies have reported that the presence of high concentrations of sialic acid residue in the sperm membrane may reflect normal spermatogenesis and maturation status of sperm.[85,86] Similarly, in both the experiments, the association between histone retention and charged sperm was observed and the removal of glycoprotein after DGC facilitates the discrimination of mature sperm (which retain some of their negative potential) from immature sperm (which completely lose their negative potential) resulting in positively or neutrally charged sperm. These results suggest that sperm retaining negative charge after DGC are likely to represent a mature population, whereas the correlation identified between histone retention and positively charged sperm supports the idea that the negatively charged sperm are more mature than the positively charged sperm.

2. *Association of sperm charge with DNA damage:* The percentage of sperm with DNA damage was inversely proportional to the percentage of negatively charged sperm and directly proportional to the percentage of positively charged sperm. Similar results were observed when Simon et al.[3] categorized the patients into three groups based on the level of sperm DNA damage, where the percentage of negatively charged sperm was higher in the low DNA damage group while the percentage of positively charged sperm was higher in the high DNA damage group. The observed correlation between sperm charge and DNA damage was low at electrophoretic field settings (6, 8, and 10 mA) and the association grew stronger with an increase in current.[35] When the sperm population was corrected to neutrally charged sperm, the correlation coefficient between DNA damage and charged sperm (negative and positive) became stronger at the lower electric field, suggesting an uniform correlation at all electrophoretic settings.

   A strong correlation between sperm charge and DNA damage suggests that loss of glycoprotein adhered to sperm membrane facilitated by the loss of membrane integrity during the process of apoptosis,[74,75] thereby causing a loss in negative membrane charge. In accordance with this hypothesis, negatively charged sperm selected through microelectrophoresis showed low DNA damage compared with positively charged sperm. These results may show that negative surface charge may be an appropriate biomarker for selecting nonapoptotic sperm without compromising the structural and functional ability of the sperm.
3. *Association of sperm charge with ART outcomes:* The charge of the sperm measured by microelectrophoresis was not associated with any of the semen parameters or men's age. The percentage of negatively charged sperm in the ejaculate was positively associated with IVF fertilization rate, whereas the percentage of positively charged sperm was negatively associated with fertilization rate. However, no correlation was observed between the sperm charge and ICSI fertilization rate.[3] This study also showed that the percentage of negatively charged sperm following DGC was positively associated with the percentage of embryos that developed to blastocyst and inversely associated with the percentage of arrested embryos. An inverse association was observed between the percentage of positively charged sperm and embryo quality. Interestingly, implantation rate was higher in the patient group containing greater than 15% negatively charged sperm after DGC compared with the patient group containing less than 15% negatively charged sperm. Couples achieving a successful clinical pregnancy had a higher percentage of negatively charged sperm and a lower percentage of positively charged sperm than those couples who did not achieve clinical pregnancy. These associations between ART outcomes and charged sperm population favor an increase in the negatively charged sperm for a successful ART outcome. Careful selection of sperm with highly negative charge using microelectrophoresis could aid in the isolation of mature and genetically fit sperm for assisted reproductive treatment.

Conclusion

Microelectrophoretic sperm selection is designed to use the sperm negative surface charge as a biomarker to identify healthy sperm. The advantages of this method of sperm selection are that it is extremely versatile, easy to use, not time consuming, does not require complex instruments, and does not require additional qualified technicians. Another clinical advantage of this approach is that the sperm selected could be directly used for ICSI insemination. Selection of sperm with a high negative charge may serve as a noninvasive biomarker for selecting nonapoptotic sperm. Although the results show that negatively charged sperm are relatively free of DNA damage, this proof-of-principle has yet to be confirmed in the context of assisted reproductive therapy and the management of male infertility.

Conclusion and Future Implications

The current methods used for sperm selection are far from perfect and a need for novel alternative methods has been emphasized to improve ART success.[2] Nearly half of the best quality sperm selected for ICSI are shown to have DNA abnormalities.[87] Therefore, the conventional sperm selection based on

motility and morphology alone are inefficient to identify healthier sperm.[11] In recent years, several new methods of sperm selection have been described to identify and select healthier sperm. These novel methods are focused on biomarkers such as sperm surface charge, apoptotic markers (Annexin V labeling), ultra sperm morphology (IMSI), and sperm membrane maturity (HA) to select healthier, more mature, nonapoptotic, and morphologically normal sperm free of DNA abnormalities.

In this chapter, we described three methods of sperm selection that utilized sperm surface charge as a biomarker to select healthy sperm. There are some inconsistencies within these methods as to whether ART outcomes may be improved by selecting sperm based on negative surface charge. The sperm selected by electrophoretic separation is shown to reduce DNA damage[52,59]; however, a clinical trial using the same group did not suggest any improvement in ART outcomes when compared with DGC.[61] On the other hand, studies using the Zeta test have reported improved ART success.[53,16] An indirect correlation between the percentage of negatively charged sperm and ART outcome by Simon et al.[3] favors negatively charged sperm to improve ART outcomes. The principal difference within these methods is that electrophoretic sperm separation methods utilize raw semen,[59] whereas the Zeta and microelectrophoresis methods use DGC washed sperm to select negatively charged sperm.[14,35] Although the process of DGC wash has been shown to induce oxidative DNA damage,[88] the process facilitates partial removal of negative surface charge from the sperm surface.[35] Partial removal of the negative charge by DGC may discriminate highly mature sperm, which retain some of their negative potential from immature sperm, which completely lose their negative charge, resulting in positive or neutrally charged sperm.[35] The group also reported that most sperm in raw semen are negatively charged (94%), whereas only 55% of the sperm retain their negative charge following DGC and such sperm retaining a negative charge after DGC are likely to represent a mature and healthy population. Based on this evidence, it can be suggested that careful selection of sperm with a high negative charge after DGC could aid in the isolation of mature and healthy sperm for ART.

Here we provided a detailed description of the quality of sperm selected based on surface charge. All three methods are able to isolate mature sperm relatively free of DNA damage. In addition, sperm selected based on charge are alive, with normal morphology and genotypically with a 1:1 ratio of X- and Y-bearing sperm.[60] Experimentally, the quality of the sperm selected based on negative surface charge was shown to be fit for ART. It is also important to note that healthy babies have been reported to be born following the use of sperm selected based on negative surface charge through electrophoretic separation and the Zeta test. The evaluation of the quality of the sperm selected by all three approaches, the clinical trial performed using the Zeta test, and the correlation of charged sperm with ART outcomes using microelectrophoresis all suggest that the selection of sperm with negative surface charge could improve ART success.

The available research and evidence regarding the charge-based sperm selection techniques still remains preliminary in nature. The two clinical trials performed using electrophoretic sperm separation[61] and Zeta test[53] were of small sample size. The methodologies of electrophoretic sperm separation, Zeta test, and microelectrophoresis vary in terms of instrumentation, where the Zeta test is fairly inexpensive and does not require complex instruments compared with others. Electrophoretic sperm selection methods are relatively quicker compared with Zeta and microelectrophoresis methods, where the latter methods involve a combination of charge-based selection step with conventional (DGC) sperm separation, which makes the methods elaborate and more time consuming than currently used conventional sperm preparation methods. The use of DGC prior to charge-based sperm selection during Zeta and microelectrophoresis methods may induce oxidative DNA damage to sperm.[65] An increase in the processing time during the charge-based sperm selection may result in a prolonged exposure of sperm to nonphysiological conditions, which may induce oxidative stress.[89]

A noninvasive method of sperm selection, without compromising sperm structural and functional ability, is essential for ART. To date, there are only two approaches known to be noninvasive methods of sperm selection: charge-based methods (described in this chapter) and the use of Raman spectroscopy.[90] These approaches have resulted in the selection of healthier sperm,[91] however, it should be noted that most of these studies are underpowered and there are no sufficient clinical data to evaluate the importance of these noninvasive selection methods. Additional research is needed to identify the group of patients who are likely to benefit using these approaches, the safety and efficacy of these approaches, and the benefits of these methods in ART. Healthy babies born following charge-based sperm selection

methods evidence of the safety of these methods in ART, but more randomized controlled clinical trials and long-term follow up of the children born are needed to support the efficacy of these approaches.

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The Relevance in Reproductive Success of Sperm Head Polarization (Birefringence)

Andrea Garolla, Alberto Bottacin, Ilaria Cosci, Massimo Menegazzo, and Carlo Foresta

Introduction

Infertility is a condition found in up to 15% of couples of reproductive age. Up until the late 1970s, there were few options available for treating these couples. Since the first successful in vitro fertilization (IVF) was described, the efficacy of subfertility treatment has greatly improved. However, it was soon realized that the technique had great limitations in achieving pregnancy in couples with compromised semen parameters.[1] It has been estimated that infertility can be caused by disorders in both the male and the female partner in 39% of couples, but male factor is considered the sole cause in approximately 20% of cases.[2,3] Currently, male infertility is evaluated through routine semen analysis; in particular sperm concentration, count, motility, and morphology are mostly considered. The introduction of the intracytoplasmic sperm injection (ICSI) technique in assisted conception has substantially reduced the quality threshold of semen in terms of concentration, motility, and morphology of spermatozoa that are necessary for a patient to be treated. In fact, by using ICSI the pregnancy can be achieved also in couples with severe male factor infertility. However, despite 20 years of technological improvements, both clinical pregnancy and live birth rates remain relatively low at approximately 35% and 25% per started cycle, respectively[4] In fact, the capacity of an apparently normal, motile spermatozoon to generate a viable embryo remains related to the incidence of abnormalities in the sperm inner structures, including altered chromatin condensation,[5,6] DNA fragmentation,[7,8] and chromosomal abnormalities,[9–11] as well as the inability to induce oocyte activation.[12]

The frequency of these defects increases proportionally with the severity of the male factor, implying the need for more effective techniques able to support the selection of fertilizing spermatozoa without altering their viability. However, current methods evaluating the sperm DNA status are invasive for cells, and thus they cannot be used to select the sperm for ICSI use. These considerations have stimulated an increasing interest in defining effective tools capable of selecting the best spermatozoon to be injected. Due to the small dimensions of the sperm cell, a meticulous analysis of its structure requires the use of a high-magnification microscope, preferably compatible with a comfortable handling of the cell to be injected. On the basis of this consideration, new and noninvasive methods of sperm evaluation and selection have been proposed to retrieve the best living sperm. In 2001, Bartoov et al.[13] suggested the use of the motile sperm organelle morphology examination technique (MSOME) based on the real-time evaluation of sperm under a magnification up to 6300×.[14,15] This method is based on the evaluation of a single sperm by high-magnification microscopy to detect the presence of minor anomalies and nuclear vacuoles, which are not visible at the standard magnification used for ICSI (400×).

Human sperm vacuoles were first described as “nuclear holes” when examined by electron microscopy and two-dimensional (2D) imaging.[16] Due to higher resolution techniques and technical progress in microscope imaging, it was recently shown that vacuoles are not nuclear holes but concavities extending from the surface of the sperm head to the nucleus.[17–19] The origin and consequences of sperm head vacuoles are still subject to controversy. It has been suggested that vacuoles originate from spermatogenesis impairment or abnormal maturation during male genital tract transit or acrosome modification during

the acrosome reaction.[20] The latter hypothesis has been explored by assessing vacuole parameters after induction of the acrosome reaction. A decreased presence of vacuoles was observed, but a significant number was still present after acrosome reaction.[21,22] Independent by their size, vacuoles seem relatively common in the sperm heads from fertile and infertile men with normal or abnormal semen parameters.[19] However, some authors observed a strong relation between the presence of large nuclear vacuoles and the impairment of sperm chromatin condensation,[23,24] which is a mandatory process involved in protection of the paternal genome before fertilization and in the early phases of embryonic development.[25] Another microscopic approach aimed at selecting the best sperm is based on the application of polarization light microscopy. This technique is based on the birefringence characteristics of the cells due to anisotropic properties of their protoplasmic texture. Most biological structures exhibit some degree of alignment that is characteristic of their molecular architecture, such as membranes and filament arrays. A membrane is modeled as a sheet of lipid molecules in which proteins are embedded, allowing the maintenance of some degree of orientation with respect to the membrane plane. Hence, tissues, cells, and organelles that include extensive membranous structures such as mitochondria and nucleus exhibit birefringence (anisotropy) as a characteristic of their normal molecular architecture. In addition to membranes, all cells and tissues include filaments that are anisotropic, such as collagen fibrils, stress fibers made of filamentous actin and myosin, and microtubules.

It is well established that when a single ray of polarized light passes through the nuclear structures of a well-ordered cell, it is refracted into two polarized rays traveling at different speeds. The difference between these phases, otherwise referred to as retardance,[26–28] causes the phenomenon birefringence. Polarized light microscopy provides a sensitive tool to analyze the alignment of molecular bonds or fine structural form in cells and has been used largely to visualize many biological structures. But only recently has sperm been evaluated with this method. Using transmission electron microscopy, some authors had previously demonstrated that sperm heads also exhibit birefringence. This phenomenon is related to the sperm nucleus and acrosome molecular order within nucleoprotein filaments that are oriented longitudinally; therefore, viable human spermatozoa are naturally birefringent, whereas in pathological conditions, dead, necrotic spermatozoa are devoid of birefringence due to the absence of conventional sperm texture.[29,30]

Some studies have analyzed the status of sperm DNA integrity in relation to the patterns of birefringence. A significantly higher incidence of DNA fragmentation was reported in spermatozoa without birefringence compared with those with a birefringent head[31,32]; moreover, birefringence has been related to a normal process of chromatin condensation.[27,28] Recently birefringence analysis has been proposed to distinguish between acrosome-reacted and nonreacted sperm cells without affecting sperm viability. In this chapter, we discuss methods, reproductive outcome, and clinical significance of sperm evaluation and selection using birefringence analysis.

Evaluation of Sperm Birefringence

In general, the traditional polarized light microscope differs from a standard transilluminating microscope in that it includes a polarizer and a compensator before the condenser and an analyzer behind the objective lens.

Most light sources (halogen bulb, arc burner, light-emitting diode) generate unpolarized light; hence the first polarizer located before the condenser optics polarizes the light that illuminates the specimen. The second polarizer serves to analyze the polarization of the light after it passes through the specimen. In its most basic configuration, the polarizing microscope has no compensator so the polarizer and analyzer are in orthogonal orientation such that the analyzer blocks (absorbs) nearly all the light that has passed through the sample. In this configuration, the image of the sample looks dark, except for structures that are birefringent or otherwise optically anisotropic and appear bright against the dark background.[33] So polarized light enables structures with molecular order to be observed when a single ray of polarized light is refracted into two polarized rays traveling at different speeds. The difference between these phases is otherwise referred to as retardance (Figure 16.1).[26–28] The effect of retardance at polarized light is microscopically evident because cells become brilliant (birefringent) in contrast with

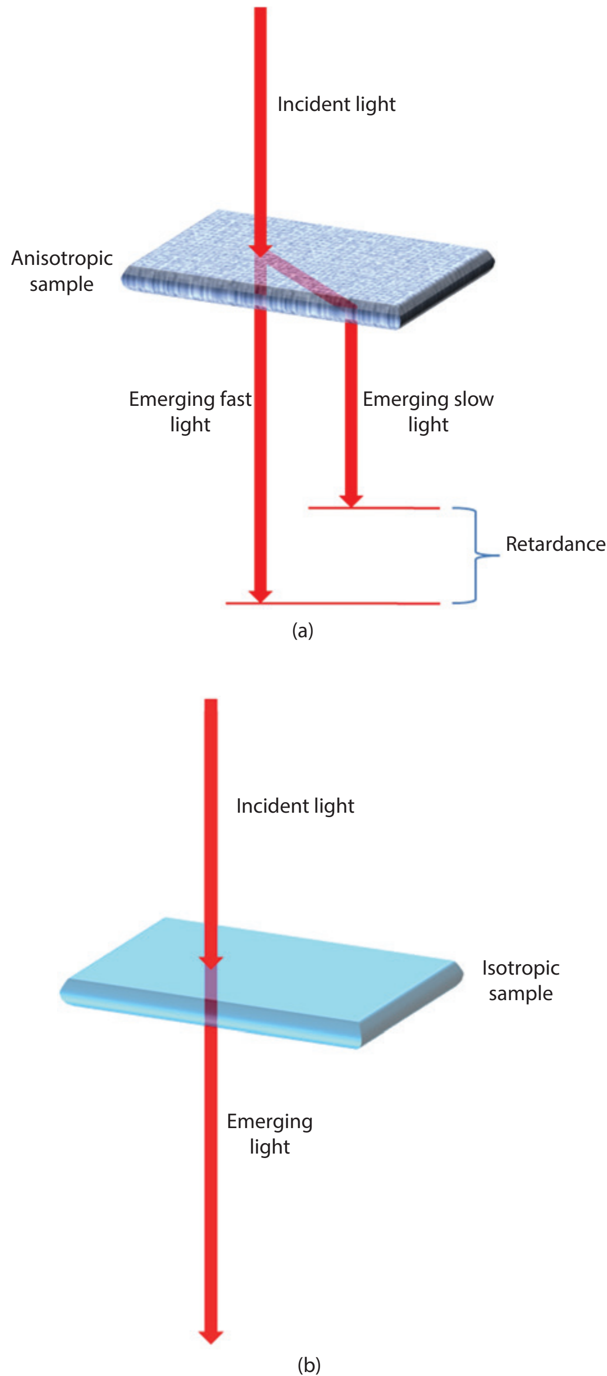


FIGURE 16.1 The mechanism by which the polarized light enables structures with molecular order (anisotropic) to refract light into two polarized rays traveling at different speeds (a). Structures with molecular disorder (isotropic) do not refract light lacking the retardance effect (b).

the dark background. Nonbirefingent cells appear as shadows. The phenomenon of sperm birefringence can be assessed by using an inverted microscope equipped with Hoffman contrast, polarizing and analyzing lenses (Figure 16.2).

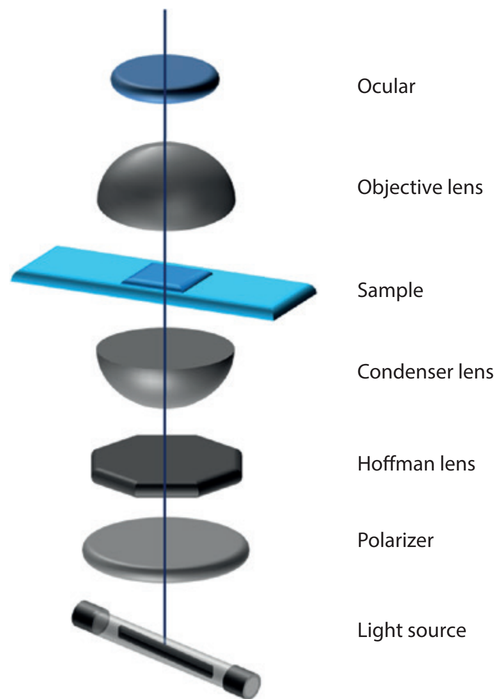


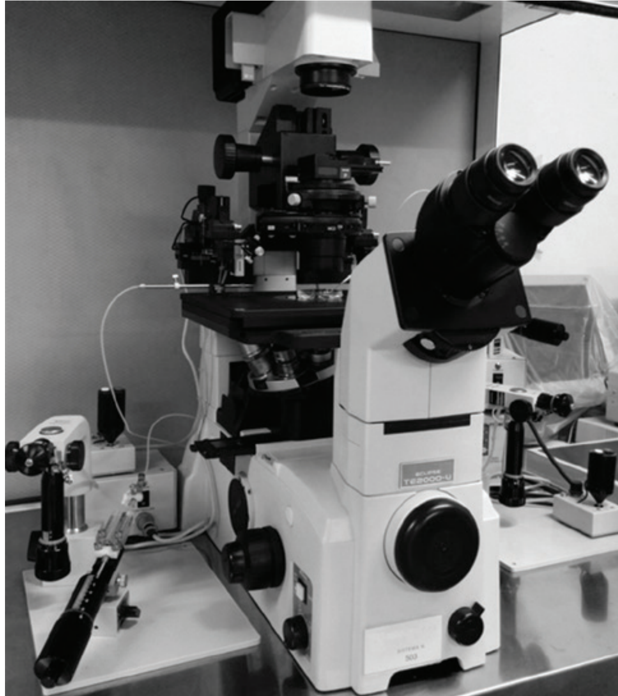
FIGURE 16.2 All the components of an inverted microscope equipped with contrast Hoffman, polarizing and analyzing lenses.

The source of light crosses the polarizing lens, the Hoffman lens, the condenser, and the specimen through a 63× objective as shown in Figure 16.3a.[26] After crossing the objective, the beam of polarized light goes through a compensator and an analyzer, entering the first optical unit that is made by a lens forming the image and a transmission prism. The resulting ray hits a mirror and is reflected along a second optic pathway through which the polarized image of the specimen is formed and then enters the ocular. The images are transmitted to a camera connected to a monitor and are captured in a computer. The microscope can be equipped with motorized micromanipulators to retrieve cells selected on the basis of their birefringence as shown in Figure 16.3b.[24]

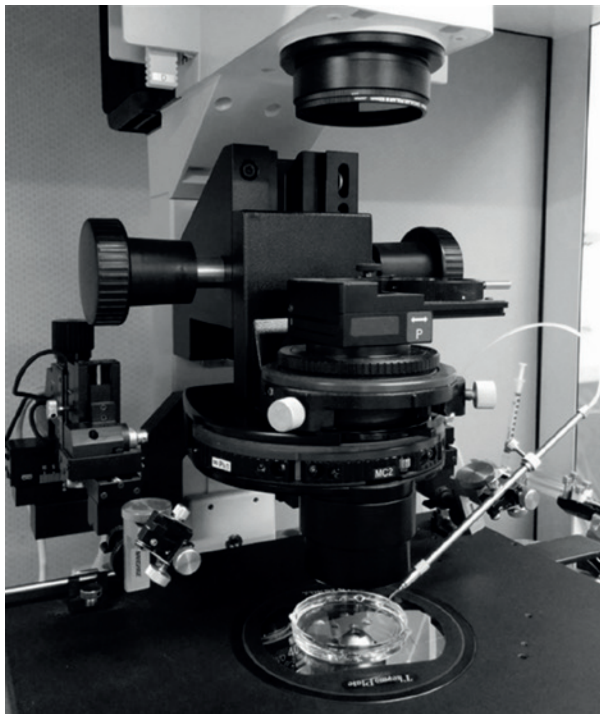
To process the samples, semen is collected in sterile containers by masturbation after 2–5 days of sexual abstinence. Semen analysis includes the evaluation of the following parameters: sperm concentration, volume, motility quality, and viability according to the 2010 World Health Organization reference values. The liquefied fresh semen samples have to be prepared using the swim-up method, which consists of permitting the sperm sample ejaculates to migrate in Sperm Washing Medium in the proportion of 1:1 deposited on top of fresh semen sample for 45 minutes at 37°C. The portion of motile spermatozoa is resuspended in this medium and the concentration is adjusted to 1×10^6 spermatozoa/mL. To analyze the sperm head birefringence using the inverted microscope equipped with Hoffman contrast and polarizing lenses, 1 µL prepared spermatozoa is incubated with 10 µL microdrop of 7% polyvinylpyrrolidone solution in a plastic petri dish and covered with oil. Three types of sperm can be identified as shown in Figure 16.4. At least 200 cells per each sample should be analyzed by two different operators.

Recent studies suggest birefringence analysis and MSOME evaluation at the same time. The microscope is equipped with motorized micromanipulators so that the sample is also ready to be used for the MSOME analysis. In this way, it is possible to look for the best sperm to be injected with ICSI, which shows birefringence according to the MSOME criteria.

Using this analysis, sperm are classified as normal when they exhibit a normal nucleus, acrosome, postacrosomal lamina, neck and tail, and do not present cytoplasm around the head. For the nucleus, the



(a)



(b)

FIGURE 16.3 Nikon Eclipse TE2000-U equipped with contrast Hoffman, polarizing and analyzing lenses (a). The same microscope equipped with motorized micromanipulator system (b).

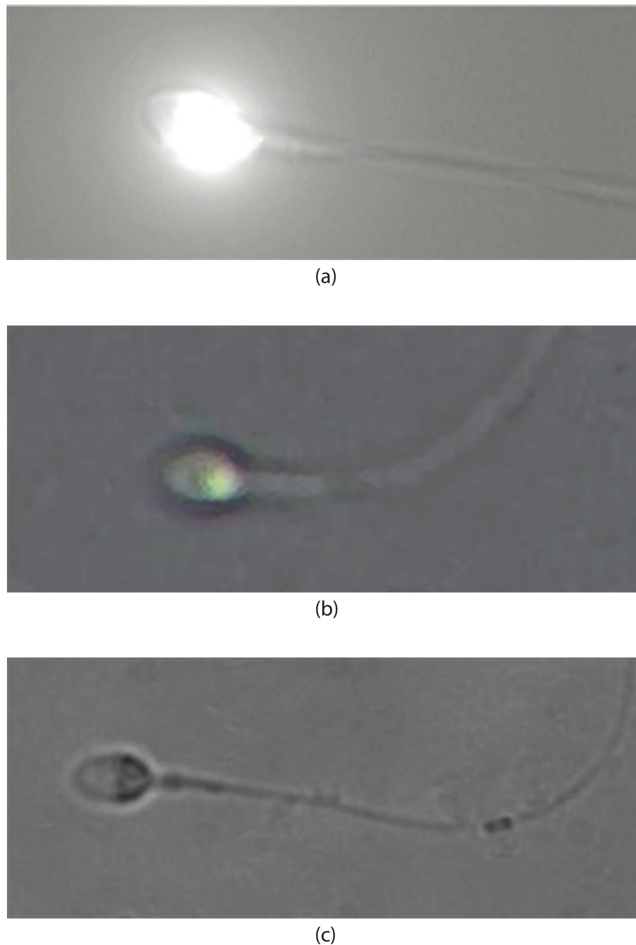


FIGURE 16.4 Different patterns of sperm birefringence: entire head birefringence (a), birefringence localized in the postacrosomal region (b), and nonbirefringence (c).

morphologically normal state is defined by the shape and presence of birefringence. The criterion for normality of nuclear shape is a smooth, symmetric, and oval configuration. Moreover, an important criterion for normality is the absence of vacuoles occupying >4% of the sperm nuclear area.[34] Vacuoles could be detected in both reacted and nonreacted spermatozoa, as well as in birefringent and nonbirefringent sperm cells.[26]

Clinical Significance

Recent studies have analyzed the status of DNA integrity in relation to the patterns of birefringence. A significantly higher incidence of DNA fragmentation was reported in spermatozoa without birefringence compared with those with birefringent heads.[31] In particular, DNA fragmentation was found to be more frequent in cells with total birefringent heads compared with those with partial birefringence.[34] In fact, this could explain the results obtained by a study that reports a higher embryo implantation rate when oocytes were injected with acrosome-reacted spermatozoa compared with those injected with acrosome-nonreacted spermatozoa (39% versus 8.6%).[26]

The relationship between the pattern of birefringence and the acrosome status was based on transmission electron microscopy (TEM) results for which it was proposed that partial birefringence was due to an already occurred acrosome reaction. It is clear now that a pattern of partial birefringence is a

very strong predictor of a reacted acrosome (96% of cells showing a partial birefringence had a reacted acrosome).

The possibility of identifying the acrosome status has important clinical implications. In fact, as already pointed out, there are data reporting that reacted spermatozoa are associated with a higher development of ICSI embryos.[35] This observation suggests that sperm undergoing acrosome reaction probably have a better genetic pattern.

Recent studies have demonstrated the association between sperm head birefringence and DNA integrity,[31,34] providing additional information about the sperm capacity to sustain fertilization and further development after ICSI. Therefore, anomalies of sperm chromatin packaging and incomplete nuclear remodeling occurring during spermatogenesis could be associated with birefringence patterns, as expressed by the negative correlation between birefringence and DNA fragmentation.[31] In addition, DNA fragmentation is significantly more frequent in spermatozoa with total birefringence compared with those with partial birefringence,[34] which are spermatozoa having a reacted acrosome and a higher capacity of giving rise to implantation.[26] These findings altogether with other studies confirm that damage in DNA structure could negatively affect the sperm capacity to undergo the acrosome reaction and the consequent steps following its entry into the oocyte.

In this light, the evaluation of birefringence properties becomes important to select sperm as it permits the identification with strong approximation of cells having a reacted acrosome and thus the highest chances of DNA integrity, which are prerequisites for further embryo development. In view of these considerations, the study of sperm head birefringence seems to provide an extra tool that, along with the characteristics of motility and morphology, predisposes the best conditions for the selection of a competent spermatozoon.[36]

A recent study by our group showed that when using birefringence or MSOME alone, there was a more than 30% probability of selecting sperm with altered DNA integrity at TUNEL test (35.2% and 37.1%, respectively). Combining both techniques, the percentage of altered sperm was reduced to 26.1%.

Interestingly, when birefringent sperm with normal MSOME and no nuclear vacuoles are considered, a significantly lower percentage of sperm with altered DNA is shown (2.1%), suggesting that the best predictor of DNA integrity is represented by the combination of these selection criteria.

Data from a recent study seem to confirm the importance of using both the techniques in the selection of the single sperm with normal DNA, aiming to obtain better results in ICSI procedures.[32] Although larger studies evaluating ICSI outcome are needed to confirm these results, it is recommended combining birefringence and MSOME by using a single microscope and privileging cells with no nuclear vacuoles present to select sperm with a higher chance of intact DNA.

Reproductive Outcome

Because it has been shown that sperm selected by MSOME give higher pregnancy and reduced abortion rates,[37] many authors reported improved clinical outcomes following the application of this technique.[38–40] In 2008, our group demonstrated that using the same method at a higher magnification (13,000×), selected sperm had better mitochondrial function, chromatin structure, DNA integrity, and aneuploidy rate.[24] Moreover, when sperm were selected on the basis of nuclear vacuoles, a further improvement of mitochondrial and nuclear status was found in sperm with no vacuoles. In particular, comparing morphologically normal sperm presenting nuclear vacuoles and sperm without vacuoles, we found the following results: altered mitochondrial function 52.2 ± 14.7 versus 13.3 ± 4.9, altered chromatin heterogeneity 71.9 ± 11.1 versus 5.3 ± 3.0, and sperm DNA fragmentation 40.1 ± 11.6 versus 9.3 ± 4.8, respectively. Finally, sperm aneuploidies were 0% in absence of vacuoles and 5.1 ± 3.1 in vacuolated sperm.

A clinical study by Wilding et al.[41] demonstrated that the pregnancy rate of patients undergoing intracytoplasmic morphologically selected sperm injection (IMSI) was significantly increased with respect to the ICSI controls. Among the 122 patients in whom IMSI was performed, 80 achieved pregnancy (65.6%). In contrast, of the 110 patients in whom normal ICSI was performed, only 44 achieved pregnancy (40.0%). The implantation rate of embryos created with IMSI procedures was also greater than

those created with standard ICSI techniques. Of the 355 embryos replaced after IMSI, 86 were implanted (24.2%). Another study on IMSI cycles showed significantly higher implantation (4 of 33, 12.1% versus 18/47, 38.3%, $p = 0.026$) and pregnancy (4 of 29, 13.8 versus 18/30, 60.0%, $p < 0.001$) rates compared with ICSI,[42] and suggested that the selection of morphologically normal spermatozoa could have a positive impact on embryo viability.

Moreover, the analysis of birefringence has been used to expand the criteria of sperm selection. Recently, Gianaroli et al.[26] also reported higher percentages of good embryos on day 3, higher implantation rates, and higher competence to progress at least beyond 16 weeks' gestation in ICSI cycles when birefringent sperm were used compared with conventionally selected sperm. The results suggested that the differences in the clinical outcome probably depend on the type of sperm samples. Patients with normospermia and those with oligoasthenoteratospermia with progressive motility yielded similar rates of pregnancy, ongoing pregnancy, and implantation irrespective of the selection of birefringent spermatozoa under the polarizing light during ICSI. However, in the categories with the most severe male factor condition, oligoasthenoteratospermic without progressive motility and testicular sperm extraction (TESE), the clinical outcome was superior when birefringence sperm were used. Moreover, a higher embryo implantation rate (39% versus 8.6%) has been reported when oocytes were injected with acrosome-reacted spermatozoa compared with those injected with acrosome-nonreacted spermatozoa. A possible explanation for this observation is that the DNA damage could alter the special cellular functions of human spermatozoa and lead to diminished acrosome reaction with reduced fertilization rates.[26] Interestingly, in the group in which the type of injected spermatozoa was mixed, the implantation rate (24.4%) was still superior to that detected in the group of nonreacted spermatozoa (8.6%, $p = 0.048$). The delivery rate per oocyte pickup followed the same trend, suggesting that spermatozoa that have undergone the acrosome reaction seem to be more prone to supporting the development of viable embryos.

These observations are in agreement with those of previous studies, suggesting that the induction of the acrosome reaction in human spermatozoa is associated with an improved fertilization outcome and embryo development.[35,43,44] Accordingly, ultrastructural studies have reported that the acrosome reaction occurs in the ooplasm before sperm incorporation in the mature human oocyte, and is preceded by acrosome swelling and followed by exposure of the inner membrane as observed on the surface of the zona pellucida during conventional IVF.[45]

Conclusions

Infertility is a common problem in the world's population and it is estimated that male factor infertility is present in approximately half of all infertile couples.[46] In this context, the term ''male factor infertility'' does not represent a defined clinical syndrome but rather an assortment of different conditions having varying etiologies and prognoses.[47] For this reason, it is impossible to define with absolute certainty when a man is fertile or infertile, so the debate is ongoing about which criteria should be adopted to define normal spermatozoa and which classification of abnormal forms is most correct to predict the fertilizing capacity of male gametes. In assisted conception cycles, the introduction of the ICSI technique has substantially decreased the threshold of requirements in terms of concentration, motility, and morphology of spermatozoa for fertilization, but at the same time it has bypassed the natural selection. Moreover, despite 20 years of technological improvements, both clinical pregnancy and live birth rates of ICSI remain relatively low.[1] Sperm quality is fundamental because it has an effect not only on the ICSI outcome but also on the incidence of embryonal abnormalities and chromosomal errors, which increase proportionally to the severity of the male factor condition.[9,11,14] All the current methods evaluating the sperm status are invasive for cells and thus cannot be performed on spermatozoa before their injection into the oocyte. Recently, noninvasive techniques of sperm selection have been proposed, aiming to better predict ICSI outcome.[15,48,49] In particular, MSOME and birefringence analysis have been reported to give higher pregnancy and reduced abortion rates.[24] MSOME is based on a morphological analysis of isolated motile spermatozoa in real time at high magnification (up to 6600×). It is able to identify not only conventional morphological sperm alterations but also more specifically sperm head vacuoles,

considered by Bartoov et al. as nuclear defects. The MSOME method has been applied to sperm injection, giving rise to IMSI. The first publications documented an increase in the pregnancy rate using IMSI compared with ICSI.[13,15] The impact of normal nuclear morphology in sperm on ICSI success has been highlighted,[37] whereas the impact of sperm vacuoles observed with MSOME in assisted reproduction technology (ART) has been questioned.[48] The variable presentation of sperm vacuoles (size, number, localization, and frequency), their mode of occurrence, their biological significance, and their impact on the quality and fertilization ability of human spermatozoa have been described. Several studies have tried to characterize sperm vacuoles, determine the sperm abnormalities associated with the presence of vacuoles, test the diagnostic value of MSOME for male infertility, or question the benefits of IMSI. Approximately 10 years after the introduction of the MSOME and IMSI procedures, there are no clear answers to several questions concerning sperm vacuoles. Several hypotheses remain unresolved: the origin of vacuoles, their relationship with the acrosome/DNA fragmentation/chromosome content, their use in male infertility diagnosis, and their impact on ART. Fundamental research on vacuolated spermatozoa and clinical prospective trials comparing MSOME with classical semen analysis, or IMSI with ICSI, are absolutely necessary to optimize the use of this high-magnification observation system. In this context, a new method, the analysis of birefringence in sperm cells, was proposed by Baccetti et al., for normal sperm selection. The birefringence within the acrosome and sperm nucleus is created by molecular order within nucleoprotein filaments oriented longitudinally and is the expression of normal organized and compact texture in cells. These observations have been confirmed by TEM. The data from the first study about birefringence and sperm confirm that the presence of birefringence in the sperm head might reflect the good health of the cell because the proportion of birefringent spermatozoa varied significantly in relation to the sample concentration, vitality, and motility.[24,29] This correlation was directly proportional to the quality of the sperm sample, suggesting that the birefringence in human spermatozoa appears to be disturbed in pathologic sperm samples, in which the inner protoplasmic structures are also affected. Then it was postulated that a benefit could be derived in terms of oocyte fertilization, development, and implantation when performing ICSI using an inverted microscope equipped with polarizing and analyzing lenses, for the birefringence analysis.

Recent studies have analyzed the status of DNA integrity in relation to the patterns of birefringence. A significantly higher incidence of DNA fragmentation was reported in spermatozoa without birefringence compared with those with birefringent heads.[32] Moreover, this analysis has been used to expand the criteria of sperm selection. In fact, birefringence analysis is able to distinguish between reacted and nonreacted sperm cells without affecting their viability.[36] The relationship between the pattern of birefringence and the acrosome status was based on TEM results by assuming that when the acrosome reaction takes place, the local protein organization disaggregates and the corresponding birefringence effect in the acrosomal region is lost. To confirm this hypothesis, a direct analysis of the acrosome integrity in single spermatozoa demonstrated different patterns of birefringence.[26,36]

The possibility of identifying the acrosome status has important clinical implications. In fact, the use of reacted spermatozoa selected by polarized light on this basis of partial birefringence was associated with a higher fertilization rate and improved pregnancy in ICSI.[26,35] In conclusion, the evaluation of birefringence properties becomes important as it permits the identification with strong approximation of sperm cells having a reacted acrosome and the highest chances of DNA integrity, which are prerequisites for ICSI success. Recent findings suggest that the combination of birefringence and MSOME technique to select sperm without vacuoles shows the lowest percentage of DNA fragmentation. Although more and larger studies are needed to confirm these findings, it appears that the combination of different methods of sperm selection can be more effective in the isolation of good sperm.

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The Applicability of Raman Spectroscopy in Sperm Diagnosis and Selection for Assisted Reproduction

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Introduction

Infertility is a worldwide health-related problem, affecting a noticeable percentage of the population whose treatments have rapidly evolved since the first in vitro child was born in 1978, permitting real success possibilities to individuals who otherwise probably would have remained childless.

Nevertheless, we also need to accept that treatments are still far from being 100% effective, and limited success options are offered, since not every couple who is able to provide sperm and oocyte succeed. In addition, several attempts are needed to achieve parenthood. A number of embryos are often needed to finally conceive a child even when using gametes obtained from donors and this is the best possible reproductive scenario.[1,2]

The goal for infertile couples is having at least a healthy child at home, with the lowest medical, biological, and economical cost possible, globally in a cost-effective manner. This means that reserach must not stop until we are able to retrieve (or create) a sperm and an oocyte from the parents-to-be that always fertilize, develop as an embryo, implant, and finally result in a healthy newborn.

There are several steps in the assisted reproduction treatments that need to be improved, including medical or laboratory issues, involving or relating to ovarian stimulation regimes and protocols, endometrial receptivity assessment, embryo culture, selection systems, etc.

To some extent, sperm research has been neglected since intracytoplasmic sperm injection (ICSI) was introduced, but we firmly believe that one area with a higher potential of improvement and relevant positive impact on assistive reproductive technology (ART) outcomes is related to sperm diagnostic and selection methods.

Ejaculates often contain several millions of motile sperm, which are genetically unique due to the random selection of either maternal or paternal chromosomes or genetic recombination, making each sperm almost unrepeatable.

Passage through the male's genital tract conditions sperm reproductive performance.

Each sperm is potentially able to result in a different child, but more interestingly, from the functional point of view, we must keep in mind that selecting one sperm for reproductive purposes may lead to success, whereas selecting another may lead to failure.

Failure may include nonfertilization, embryos' arrest during their development, implantation failure, or fetal development defects resulting in miscarriages or children with health problems. In a natural conception, the mechanisms deciding which single sperm will fertilize are not fully understood, but when applying ART, sperm are to some extent subjectively selected, and in a number of cases, this is decided by the operator.

To maximize the right choice of selection of the sperm by the embryologist, two requirements need to be fulfilled: first, to establish and define the characteristics of the best sperm (or at least the good sperm) and second, to identify and handle them without harming their viability and functionality (avoiding, if possible, situations equivalent to Heisenberg's uncertainty principle that asserts that there is a fundamental limit to the precision with which certain pairs of physical properties of a particle can be known simultaneously, meaning that the analysis is somehow influencing the analyzed sample).

The means by which sperm selection methods could exert a relevant influence on ART results are caused by the possibility to extend their influence on the entire cohort of embryos obtained.

Typically, in ART success reports, results are provided per embryo transfer, but they should in fact be provided per number of embryos transferred, including fresh and frozen embryos transferred, via cumulative rates,[1] per oocyte pick up or patient, and this information is frequently lacking. Results per embryo transfer are only affected by interventions conducted on embryo transferences.

Implementing such diagnostic and/or selection techniques for sperm could result in live-birth rate improvement per patient or controlled ovarian stimulation protocol by maximizing the sperm factor contribution but still acknowledging that results will also depend on oocyte and endometrium quality.

Several sperm quality characteristics have been reported in the literature, and many of them are covered within this book. We can make a categorization between those reflecting cellular characteristics and molecular characteristics, where sperm morphology or ultramorphology is an example of the first and sperm messenger RNA (mRNA) content is an example of the second.

Some cellular characteristics in sperm have been suggested as potential quality markers as hypo-osmotic swelling test (HOST) (vitality)[3] has been demonstrated, which have also been able to identify individual spermatozoa with minimal DNA fragmentation but without traits of apoptosis, abnormal head morphology, nuclear immaturity, or membrane damage.[4]

Motile sperm organelle morphology examination (MSOME) has been developed to address one of the main concerns in ICSI, the subjective selection of spermatozoa presenting both motility and normal morphology to be microinjected, based on the embryologist's evaluation under an optical magnification of 400×, to avoid relevant sperm anomalies, without requiring fixation and staining.[5]

This magnification is not sufficient to show subtle nuclear defects, with the risk, according to some authors, of having low success rates or even transmitting genetic and chromosomal diseases to future embryos and children.[6,7]

Also, it is quite likely that the cell performances are conditioned by the molecular characteristics, but finding techniques that are able to identify molecular traits in the spermatozoa and to keep them unharmed is not that easy.

The sperm ability to bind hyaluronic acid (HA) is one, and the technology developed to remove apoptotic cells from an ejaculate is another, based on sperm membrane characteristics, permitting cell viability after these positive or negative selections.

In spermiogenesis, during sperm plasma membrane remodeling, along with the formation of zona pellucida receptors, receptors for HA are created as a symptom of sperm maturity. Displaying HA-binding capacity is supposed to be positive for a sperm cell, given that it is one of the oocyte's zona pellucida major components.

Birefringence is defined as the refraction of a ray of light into two rays traveling at different speeds when passing through anisotropic materials. In the case of spermatozoa,[8,9] their subcellular structures, including nucleus, tail, midpiece, and mature acrosomal, exhibit complex strong intrinsic birefringence generated by their molecular nature.

The application of polarizing and analyzing lenses in the inverted microscope used in the ICSI technique permits the possibility of identifying birefringent sperm cells without affecting their vitality or motility,[10] providing similar information to that obtained by the use of transmission electron microscopy and improving substantially the observation obtained by phase contrast microscope.

Mainly in infertile males, a varying percentage of apoptotic sperm cells have been described to present in their ejaculates. This was detected after the evaluation of apoptotic protein presence, leading in some cases to a significant increase in DNA damage.[11–16]

Although frequently related to other abnormalities detectable by light microscopy, it appears that other sperm cells with apoptotic features may remain normally shaped and then be candidates for microinjection escaping from morphological sperm selection.

Why these apoptotic cells originate or why they are not efficiently removed from ejaculates remains unclear but this may be linked to the process length, suggesting that in these cases the apoptosis may have been initiated at some point and not terminated yet at the moment of ejaculation.

But it appears that there multiple sperm factors related to fertility (a myriad, in fact), including sperm DNA integrity features, each with a specific positive or negative contribution. Up to now, several

molecular factors have been described individually as relevant in the reproductive process, leading to the conclusion that infertility may be caused by a multifactorial or cumulative failure in one or more cell type/tissue. This was a very complicated issue to address a decade ago, but fortunately, the advent of massive molecular analysis technologies allows us to gather a huge amount of information from every single experiment, which after proper analysis, permits a broader view of complex biological systems,[17] with relatively low cost and effort.

These -omics technologies are disciplines that include the study of the events and interactions of cellular structures and processes, including DNA and their biological functions, i.e., from DNA and genes to metabolites in a comprehensive manner.

Sperm cells are by far the most specialized cells in the human body to accomplish a very difficult mission, with successive phases, each one independent and highly critical.

After being deposited in the vagina during the intercourse, they will swim through cervical mucus, toward the uterus and tubes, reaching the oocyte to fuse with and initiate mechanisms to coordinate both maternal and paternal genomes to achieve embryo development and implantation.

The spermatozoon is divided into compartments with specific roles[18]: (1) the head containing DNA that needs to be correctly packed and unpacked at precise moments, (2) the midpiece where mitochondria will generate energy, and (3) the flagellum which transforms energy into motion.

All these functions are dependent on several molecules, whose evaluation, theoretically, could find a place in future semen analysis given their physiological importance.[19,20]

Any sperm's capacity to succeed is as weak as the weakest point through this chain of events. It appears that it is very complex to be a successful sperm, and it is even more difficult to be able to precisely measure how successful it can be.

This is the basis of the importance of sperm cell selection in assisted reproduction: success depends on one single cell.

Genomics is the study of the complete set of genes expressed in a certain cell type or tissues at a specific time. Proteomics is the study of the complete set of proteins.[21] Secretomics is the study of the complete set of proteins secreted by the cells into the surrounding medium. Metabolomics is the study of the complete set of resulting metabolites in a defined environment originated by cell metabolism. Epigenomics, recently introduced, is devoted to the study of the heritable changes in gene expression that occur without changing the gene sequence.[22]

Although the functional significance of mRNA in mature spermatozoa has been extensively debated, it is known that mRNA is necessary from the first embryo cleavages until the embryo activates its own genome. The microarrays are in a glass, silicone, or nylon matrix with anchored series of oligonucleotide probes fixed at precise locations that will unequivocally bind to specific mRNAs by molecular hybridization, fluorescence, chemiluminescence, or tagged radioactivity.[23]

It was hypothesized that the molecular requirements for semen samples to be able to achieve pregnancy are different for each ART, and there are fewer molecular requirements when the reproductive techniques are more invasive, given that ART is somehow substituting the natural process, meaning that the molecular machinery needed for sperm is unnecessary when ART is used to achieve pregnancy.[24]

From this viewpoint, we defined the different gene expression profiles for each of these techniques depending on whether or not pregnancy was achieved.

A number of specific genes, biological processes, cellular components, etc., have been demonstrated to be expressed differently depending on pregnancy status, including genes related to male fertility, and on the assisted reproduction technique used such as intrauterine insemination (IUI), in vitro fertilization (IVF), or ICSI. Interestingly, some of these genes have been found to be important and involved in reproductive results in all three ARTs, which makes them of special interest. It is important to explore their role in sperm function,[24] that they are serious candidates that need in-depth analysis.

To date, a large number of proteins have been identified that may play a role in male fertility,[25,26] specifically, sperm nuclear proteins,[27] due to the function displayed in epigenetic marking, proper fertilization, and embryo development.

Metabolome analysis exhibits some advantages compared with the previous -omics described, given that they are the end products of expression, translation, and protein modification, including protein function, and provide higher sensitivity to be used as sperm function biomarkers. Abnormal

spermatogenesis has been investigated using this approach, [28] as well as seminal plasma from fertile and infertile men.[29]

Other technologies have also emerged that are attractive and promising because of their ability to deliver results related to molecular profiles in living cells, as in the case of Raman spectroscopy.

In summary, the importance of good sperm selection methods to improve reproductive outcomes appears evident. The need for identification of fertility markers in sperm is the way one can identify good sperm. Finally, analysis techniques that ensure that spermatozoa remain undisturbed and that can be used in ART are necessary to accomplish these objectivies. Raman spectroscopy is a strong candidate that meets these criteria.

Raman Spectroscopy

Raman spectroscopy is a promising analytical tool that can be used to determine specific molecular traits in biological tissues and single cells based on physical properties of molecules while maintaining viability as a potentially interesting sperm diagnosis and selection method.

In this chapter, we will review the basis of the method together with the available bibliography on sperm and discuss the pros and cons to be implemented in routine sperm selection.

The principles underlying this technology are not new. They were described almost 90 years ago but recently have re-emerged due to improvements in optics, miniaturization, and big data analysis technology, together with several technical improvements that made it possible to deliver a detailed molecular "fingerprint" of biological samples in good time that leaves samples undisturbed.

As with other -omic techniques, its main advantage may also be considered its main problem, given the complexity and amount of data provided, where "noise" makes it difficult to find clinically relevant data, requiring multidisciplinary approaches, and complementing profiles of the biomedical researchers with other specialists in data management and analysis, chemists, etc.

Raman spectroscopy is based on the phenomenon of inelastic scattering of parts of a light source by a transparent material, which provides specific information on specific features of each molecule, which presents its own molecular vibration pattern that can be used as a kind of ID, the so-called "Raman biomarker."[30]

The Raman effect is the capacity of most photons from an incident light source to retain their energy after coming across the atomic bonds of molecules (elastic scattering). In a small percentage (of about one per million), the interaction causes changes in frequency and wavelength (inelastic/Raman scattering). These changes or shifts, typically from a few hundred to a few thousand wave numbers, vary depending on the atomic mass, quantity of valence electrons, and molecular bonds encountered, being unequivocally particular for each of the molecular constituents, their arrangement, and their state.[31] This means that each molecule has a unique and characteristic pattern.

Fine analytical techniques, with extremely high resolution, have been used to analyze organs, tissues, and individualized cells. These techniques, including, for example, analytical electron microscopy, x-ray imaging, or secondary ion-mass spectroscopy, among others, all had disadvantages. The samples were destroyed during analysis[32] and it was impossible to analyze living cells in a way that would enable subsequent use of the sample following this initial analysis.

Raman spectroscopy provides molecular information about living organs, tissues, or cells, while maintaining cell viability and keeping these cells entirely unaltered, even ready, for future use.

The Raman spectrometer that conducts this analysis is composed of a laser optically able to provide detailed information about chemical composition, molecular structures, and sample variations in living tissues without the need to add external labels or extensive preparation by means of the inelastic scattering of light behavior. This analytical tool translates any changes in cellular biochemistry into spectroscopic differences revealed by Raman spectra; the unique and particular spectra for each tissue is identified by chemical or biological mark,[31,33–36] which can be used to identify and characterize biomolecules within cells or tissues.

Over time, technical improvements have been incorporated, combined with other devices and instruments, for example, confocal microscopy, allowing the identification of molecules in organelles, thus

permitting a kind of three-dimensional (3D) spatial resolution[30,33,35–40] that can even be carried on single cells.

These tools were used initially by physicists and chemists. In the 1970s their use in biological/medical investigation became popular, and several investigations were performed[31] that resulted in the development and sophistication of Raman techniques and that expanded the scope of studies. Raman spectroscopy allowed the examination of entire cells as well as tissue sections. Tissues, pathological situations, and organ damage were identified and differentiated using the Raman properties.[38,41]

Initially, Raman spectroscopy was used in reproductive medicine for investigating cancer. Raman spectroscopy showed the molecular differences between benign and cancerous states on tissues by comparing four intensities of Raman fingerprints for cervix, uterus, endometrium, and ovary.[42]

Later studies focused on other biological samples such as breast cancer,[43] axillary lymph nodes,[44] microcalcifications,[45] and silicone implants.[46]

Use of Raman Spectroscopy in Analysis of Male Reproductive Tissues, Fluids, and Cells

The application of Raman spectroscopy analysis to cells or fluids linked to reproductive function and disease resulted in several studies to decipher their molecular characteristics to better understand the physiology of the reproductive outcomes.

For instance, testicular tissue characteristics have been analyzed, with the inherent difficulties of a tissue composed by a variety of cell types, conforming to a complex environment. An interesting initial analysis published in 2004 by De Jong et al.[47] mapped the microliths from seminiferous tubules of men with different testicular conditions, demonstrating that their main component was hydroxyapatite, and most importantly, when surrounded by glycogen, there always was an association with malignancies.

Testicular carcinoma was characterized by Raman spectroscopy[48] with the seminoma cell model TCam-2 cells, showing two types of cells, again subdivided into two groups, one with molecular similitudes to embryonic carcinoma cells.

Using the Raman technique, one group studied the animal (bovine) models of testicular cells and investigated in vitro the bovine spermatogonia leading to spermatids.[49] Other studies on human models used Sertoli cells from biopsies gathered from patients with azoospermia,[36] which showed preliminary data, but with low clinical relevance.

Seminal plasma is a fluid with key relevance to spermatozoa and their function. In the pioneering works conducted by Virkler and Lednev,[50] Raman profile studies of seminal plasma revealed a rough estimate of the presence of several key constituents in human semen such as albumin, fructose, lysozyme, lactate, and urea, among others, and smaller peaks were studied in depth. The main components analyzed were sufficient for identification in body fluids and even species.[51]

Mallidis et al.[39] conducted Raman analysis of human seminal plasma and described three regions of “overlaid signals” at 820–850, 1010–1100, and 1220–1350 cm^{-1} together with five relevant peaks at 714, 955, 1000, 1447, and 1666 cm^{-1} that were initially attributed to proteins.

The work by Huang et al.[52] specifically described that the ratio between the peaks at 1418 cm^{-1} (corresponding to a-methylene CH_2 scissoring) and 1448 cm^{-1} (tryptophan) is able to discriminate between seminal plasma from patients (or samples) with normal and abnormal sperm morphologies.

Moreover, studies attempted to relate these peaks in seminal plasma with specific sperm quality parameters within the ejaculate by using, in this case, polarized SERS (surface enhanced Raman) spectroscopy.[53]

Obviously, the final product of spermatogenesis is spermatozoa. These are the cells that finalize the process and affect fertilization and postfertilization events.

Spermatozoa are an interesting subject for these kinds of studies.

Kubasek et al.[54] tested salmon sperm extracted DNA in an approach that had not yet been attempted with human spermatozoa.

Recently, different groups have studied and described the spectra of different sperm regions[32,39,40] using the microspectroscopic form of the technique describing typical spectra for each sperm region.

Nevertheless, no clinical application or correlation was applied beyond the described. Moreover exists disagreement about a specific spectral region of the head and a variation in the ratio of the 785 cm-1/1442 m-1 peaks to predict normal morphology; which Mallidis was unable to confirm.[39]

Meister et al. described neck and mid-piece components of the sperm, suggesting that a peak at 751 cm^{-1} was compatible with the presence of mitochondria, and thus disagreeing with previous studies.[32,39]

It was sufficiently well defined that the peak at 1092 cm^{-1} was related to the PO_4 backbone of DNA and this was useful for evaluating nuclear DNA status, including damage and its corresponding location by analyzing the peak intensity and increasing the peak corresponding[39] to 1040–1050 cm^{-1}, which was either caused naturally or induced artificially.[33,55]

This was confirmed by studies with sperm bound to the zona pellucida[37] and mapping the damage, as well as analyzing it more meticulously.

Some external influences on the Raman profile for sperm have also been evaluated using Raman as a representation of sperm status.

For example, it has been investigated that Raman microspectroscopy is able to detect oxidative DNA damage in the nucleus by means of a three-way comparison of Raman profiles, Fourier transform infrared spectroscopy (FTIR) spectra, and flow cytometric assessments of sperm nuclear DNA (nDNA) damage.[33]

Changes after the induction of oxidative damage by Fenton's reaction on semen samples by both Raman and FTIR spectra were indicative of oxidative attack, given that changes in Raman profiles were similar to those previously described for the DNA backbone, even with different degrees of damage, establishing a cutoff value of 0.63 and an estimation of the percentage of sperm with nuclear DNA damage established by the ratio of peaks (1050/1095 cm^{-1}) that correlated linearly to the flow cytometric assessment. Hypothetically, this is a meaningful way to select spermatozoa with integer DNA, which is useful for the Andrology Laboratory.

Other groups studied the antioxidant protective effect of several substances such as oligosaccharides extracted from *Morinda officinalis*[38] and icariin extracted from *Herba epimedii*.[30] The researchers induced sperm DNA damage by using H2O2 or FeSO4/H2O2 and then they co-cultured these samples with oligosaccharides and icarrin. Both studies suggested that these substances can enhance the sperm oxidative strees being used as an antioxidants in male fertility improving reproductive functions. These changes produced by means the co-culture of these substances may be detected at a Raman microspectroscopy.

Li et al.[35] completed a study that aimed to evaluate the possibility of label-free, rapid identification of human sperm damage caused by maleic acid to test the effects on the regions of acrosome, nucleus, and middle piece. The use of maleic acid as a cervical or vaginal contraceptive was also studied.

They reported that Raman spectroscopy indicates significant changes in the different regions of sperm cells, suggesting destructions and conformational changes in proteins and lipids and damage to nuclear DNA and mitochondrial DNA structures.

Liu et al.[34] used this technology to differentiate seminiferous tubules with complete and incomplete spermatogenesis to help the urologist select the best tubule in microtesticular sperm extraction (micro-TESE) by scanning the human testicular tissue at different maturational stages, by immunohistochemistry study, and by metabolomic analysis of nonobstructive azoospermic (NOA)/obstructive azoospermic (OA) testes.

They demonstrated that tubules of OA patients showed spectral intensities <2000 (au), whereas tubules of NOA patients had higher ones, proportional to the degree of spermatogenesis. The test is able to discriminate with a sensitivity of 90% and a specificity of 85.7%, complementing the finding that the gas chromatography–mass spectrometer (GC–MS) showed significant differences in 12 metabolites between NOA and OA testes in a way that permits a noninvasive analysis to distinguish seminiferous tubules with complete and incomplete spermatogenesis.

There is thus a potential role for the use of this technique in this field, allowing for improvements in the rates of sperm retrieval after surgery without the use of labeling agents or invasive techniques.

Although this particular application of Raman microspectroscopy still requires further validation, it can be considered a potential diagnostic tool for reproductive medicine.

Conclusions

Raman spectroscopy has been used successfully to determine molecular features of sperm while maintaining viability, such as DNA packaging and DNA structure damage (oxidative damage) of individual spermatozoa and the integrity of subcellular organelles such as mitochondria and sperm shape and morphology.

It has also been used to evaluate spermatogenesis in human seminiferous tubules, not requiring external labeling, as well as differentiating Sertoli cells from patients with NOA or OA, together with the differences in the characteristics of sperm bound to zona pellucida and unbounded ones.

The pros of this technology include the detailed, precise, accurate, and reproducible information on sperm components, including their spatial distribution, without affecting cell integrity.

The technology is efficient, easy to operate, reliable, and efficient.

The cons of this technology include the accessibility and extremely high cost of the equipment, together with the need for a highly specialized team of experts for its management and analysis.

Raman spectroscopy is a promising diagnostic tool for reproductive medicine, especially in andrology, with exciting potential, although the clinical application still requires further assessment and strict safety evaluation.

Further investigation is imperative in this area to properly evaluate the costs as well as the benefits to infertile patients.

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The Use of Sperm Proteomics in the Assisted Reproduction Laboratory

Meritxell Jodar, Ferran Barrachina, and Rafael Oliva

The Scientific and Biological Bases of Proteomics

About 84% of general population is able to conceive after 12 months of unprotected and regular intercourse.[1] However, 9% of women aged 20–44 years are unable to achieve a live birth during this period and often seek reproductive care.[2] Today, approximately 2% of the children born in Europe are conceived through assisted reproductive technology (ART).[3] Identifying the causes of infertility is crucial to establish the appropriate clinical treatment and minimize the risk of failure. Fertility evaluation for men is currently limited to examining semen parameters, which are able to reveal gross deficiencies in sperm count, motility, or morphology.[4] However, the etiology of male factor infertility remains largely idiopathic. Nowadays, fertility treatments offer a good rate of success, and approximately three out of four women will get pregnant as a result of ART. However, couples often have to face several attempts of ART cycles, which becomes a costly and time-consuming process. In Europe, this represents 134,422 live births from the 536,886 ART cycles including in vitro ART (in vitro fertilization [IVF] with or without intracytoplasmic sperm injection [ICSI]) and intrauterine insemination (IUI) reported in 2010 from women of all age groups.[3] This is a rather sobering statistic reflecting a per-ART-cycle failure rate of ~75% that is substantially higher than that of the naturally conceiving population.[3] Although the utility of some genetic tests (cytogenetic alterations and DAZ deletion) to predict unsuccessful results for ART cycles is unquestionable,[5,6] the series of semen parameters widely used in reproductive clinics to assess the male fertility are of little correlative value with respect to pregnancy outcome.[7] This observation emphasizes the need to develop alternative strategies for more accurate assessments.[8] High-throughput technologies such as proteomics provide the expression levels of all proteins of one functional state in a biological dynamic system. The application of proteomics to the study of the spermatozoa has progressed at a fast rate over the past 20 years (Figure 18.1).[9] The results obtained are shedding new light on the different issues of the sperm biology including generation, maturation, and metabolism of the mature sperm cell capable of fertilizing the oocyte.[10] Two main processes occur during spermatogenesis: (1) the replacement of histones by protamines and (2) the expulsion of majority cytoplasm during last steps of spermatogenesis, resulting in the blockage of nuclear transcription and translation in the mature sperm cells.[11,12] Then, the proteomic studies on the transcriptionally and translationally inert sperm cells, which are unable to generate new nuclear proteins, represent the final static picture of spermatogenesis. Results derived from comparative sperm proteomics between fertile and infertile males may provide insights into pathogenic mechanisms of male infertility. To date, sperm proteomics has only been applied in the research laboratory; however, the information derived from proteomics-based studies is likely to be useful in the development of fertility biomarkers. Therefore, proteomics holds promise of utility in clinical diagnostic testing of sperm for infertility, which is currently largely limited to the analysis of seminal parameters (sperm concentration, motility, and morphology).

Proteomic Techniques

The study of sperm proteins started more than a century ago with the isolation and identification by Friedrich Miescher in 1874 of a proteinaceous basic component from the sperm cell that he called "protamine" and that he found was coupled to what he called "nuclein" or what we know as DNA.[13] However, it was not until about 100 years later that the protein sequencing, separation, and detection methods were developed allowing the generalized study of the proteins (Figure 18.1).[14–16] Nevertheless, with these methods the proteins still had to be studied one at a time. The possibility to study the entire or a substantial proportion of the sperm proteome started much more recently, around 1995, with the application of mass spectrometry to the study of proteins (Figure 18.1).

The basic steps in most proteomic analysis at present are (1) protein or peptide extraction from the biological sample, (2) reducing the complexity of the protein or peptide extract, and (3) application of mass spectrometry and database comparisons to identify the different proteins or peptides (Figure 18.2).[17] The first step as applied to the sperm cell can be accomplished either by extracting the entire sperm or fluid proteome as well as by targeting specific cell compartments such as membrane systems, nucleus, tail or organelles, or fluid components.[18] The second step or reduction of the complexity of the initial protein or peptide extract can be accomplished using one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) or 2D-PAGE (Figure 18.2). However, a more recent and high-throughput approach is to convert the initial protein extract into peptides on digestion with a protease and subsequently to fractionate the peptides using peptide isoelectric focusing (IEF) or monodimensional liquid chromatography (1D-LC) or 2D-LC (Figure 18.2).

The final step in a proteomic analysis is accomplished through mass spectrometry peptide and protein identification. Initial proteomic methods were developed that involved matrix-assisted laser desorption ionization—time of flight (MALDI-TOF), which relies on the accurate determination of peptide masses and comparison to peptide mass databases in search for identities. In a MALDI-TOF analysis, the proteins are typically excised from the gel, digested with trypsin, and the ratio of mass to charge of the resulting peptides determined. These peptide masses provide an accurate "peptide mass fingerprint" for

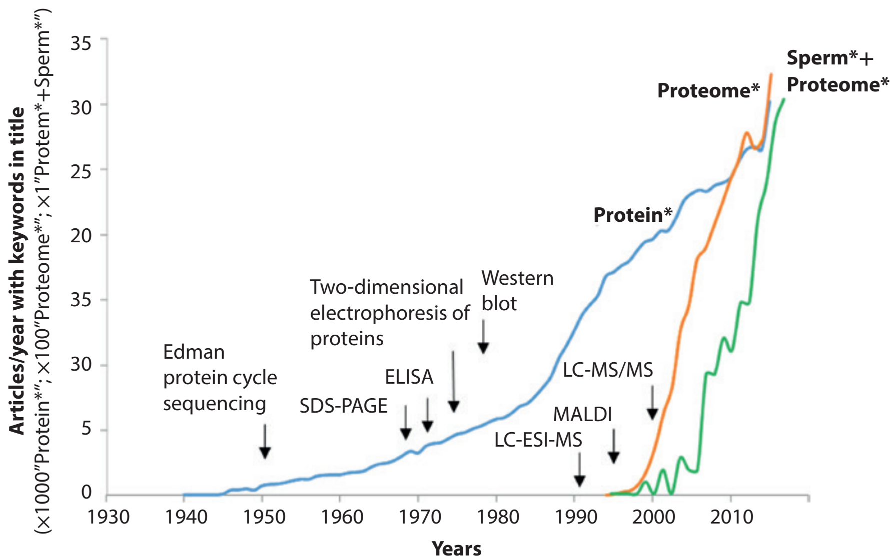

FIGURE 18.1 Pubmed publications where the keywords "protein*" or "proteome*" appear in the title. The asterisk "*" in "protein*" or "proteome*" indicates a wildcard. The year of the description of key methods to study proteins is indicated with arrows. It can be observed that proteomics is a relatively recent field as it started in 1990 with the application of mass spectrometry to study proteins.

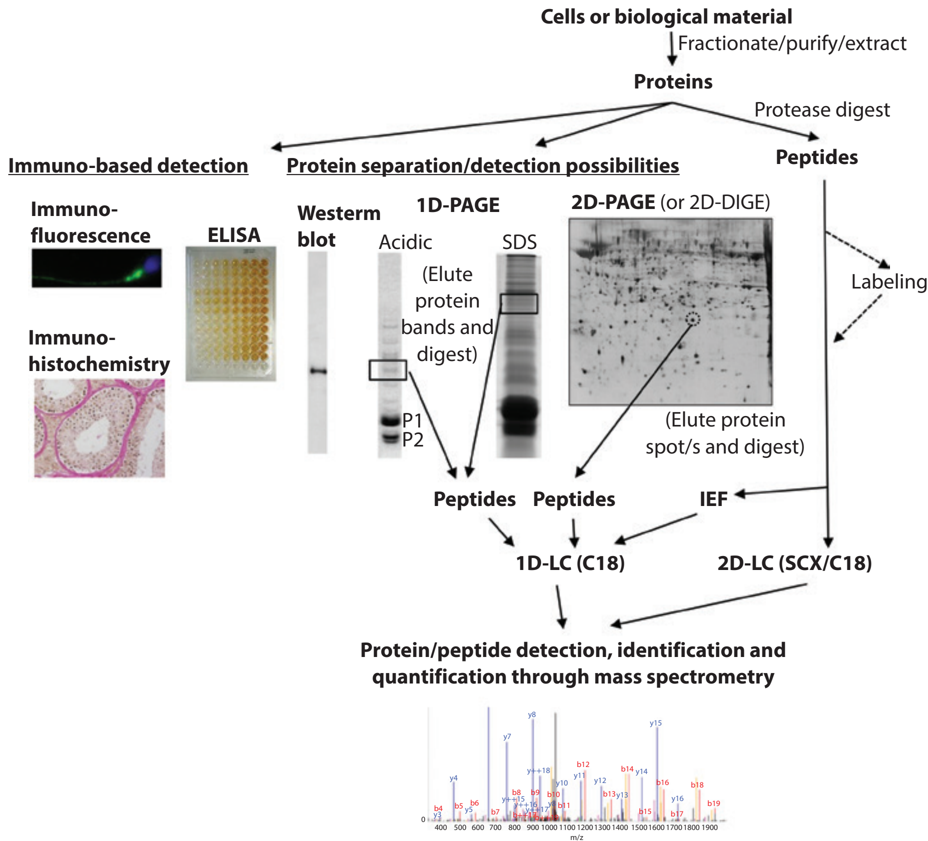

FIGURE 18.2 Many protein analysis options are currently available. Usually sperm cells or biological material must be processed, purified, or fractionated before proceeding to extract the proteins or their targeted detection. Cells or tissue sections can be directly used using immunohistochemistry or immunocytochemistry (left). Alternatively, proteins can be separated by gel electrophoresis (center) and the desired proteins eluted and digested into peptides. A current very high-throughput approach involves the digestion of the original protein mixture by proteases (usually trypsin) to convert it to peptides (right). The final stage is to separate the peptides through liquid chromatography and to proceed to identification using mass spectrometry.

the protein and are then compared against sets of masses from databases of in silico predicted peptides derived from the genome. If several of the experimentally determined peptide masses matched with the theoretical peptide matches derived from the proteins in the databases, then it is considered that the protein has been identified.[19] However, currently higher-throughput approaches based on tandem mass spectrometry (MS/MS) are being applied that also provide the opportunity for de novo peptide sequencing and posttranslational modifications detection (Figure 18.2).[17]

For protein quantification different possibilities are also available. Initial methods developed were based on enzyme-linked immunosorbent assay (ELISA)[14] or western blot[16] (Figure 18.2). These methods are extremely robust and useful but applicable only to study specific target proteins and cannot be applied to study many proteins simultaneously or even substantial proportions of the entire proteome. High-throughput approaches are currently available to quantify simultaneously many proteins in the proteome. Initial proteome quantification methods were based on measuring the protein intensities of proteins separated on 2D gels and identifying the corresponding protein spots.[20,21] However, current high-throughput quantification techniques rely on peptide quantification rather than protein quantification. Peptides can be quantified by spectral counting[22] or after their in vivo or in vitro labeling with tandem mass tags (Figure 18.2).[23,24]

Scientific Evidence

Currently, the analysis of the whole sperm proteome and subcellular proteome composition such as that corresponding to the sperm head,[25–27] tail,[26,28] and membranes[29,30] has resulted in the identification, with high confidence, of 6238 different proteins in the entire spermatozoa (Figure 18.3a).[10,18] Differential proteomics studies involving sperm cells from different subtypes of infertile patients

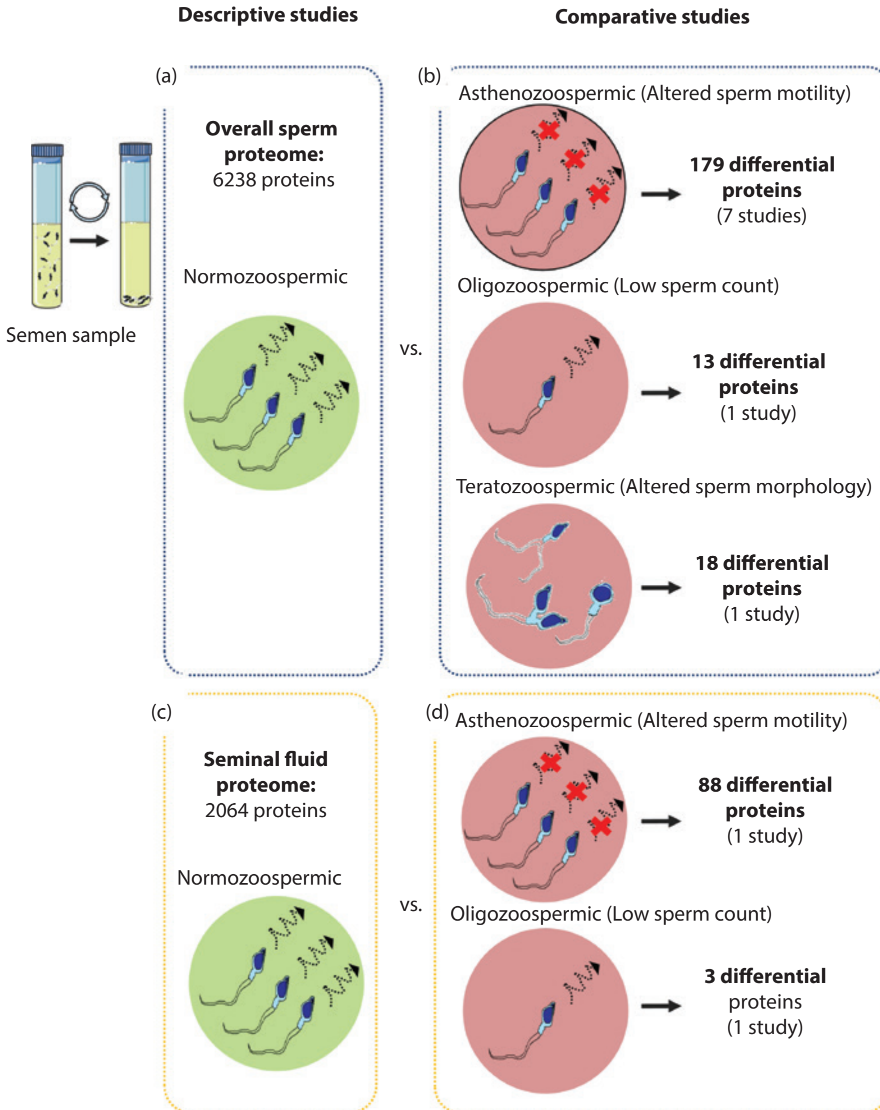

FIGURE 18.3 Descriptive and comparative semen proteomic studies according to seminal parameters. (a) A total of 6238 proteins have been identified in the sperm proteome of human normozoospermic spermatozoa. (b) Comparative sperm proteome studies between normozoospermic and different subtypes of male infertility according to their seminal parameters have revealed 179, 13, and 18 differential proteins for asthenozoospermic, oligozoospermic, and teratozoospermic patients, respectively. (c) A total of 2064 proteins have been identified in the seminal fluid proteome of human normozoospermic individuals. (d) Comparative seminal fluid proteome studies between normozoospermic and different subtypes of male infertility according to their seminal parameters have revealed 88 and 3 differential proteins for asthenozoospermic and oligozoospermic patients, respectively.

according to the seminal parameters has revealed functionally important proteins that could help to understand the various pathogenic mechanisms implicated (Figure 18.3b).[20,24,31–37] The most commonly studied sperm phenotype has been asthenozoospermia (altered sperm motility) comprising seven different studies and detecting a total of 179 differentially expressed proteins.[20,24,31–33,35,37] Most pathological mechanisms probably affecting sperm motility are based on the differential proteins detected and disturbances in the generation of energy required for sperm motility (those mainly involved in the citric acid cycle) and in the regulation of apoptosis. Spermatozoa only account for 5% of the ejaculate, whereas the remaining 95% corresponds to secretions from different accessory sex glands. A total of 2064 proteins have been identified in seminal fluid revealing that, contrary to being a simple medium to carry the spermatozoa through the female reproductive tract, the seminal fluid seems to be crucial for the regulation of semen coagulation and liquefaction, sperm motility, and fertilization[10,38] (Figure 18.3c). Comparative analysis of seminal fluid proteome from different infertile patients enables the assessment of the accessory sex glands function[39,40] and also their impact on sperm physiology.[41,42] A single study assessing differential proteins in the seminal fluid proteome from asthenozoospermic patients suggests the disturbance of processes associated with the energy production by glycolysis.[41] The glycolysis process seems to be related to the microvesicles contained in seminal fluid released mainly by prostate[43] with a known ability to produce extracellular adenosine triphosphate (ATP) from carbohydrates[44] and probably acting as an auxiliary tool to provide energy for sperm motility.[45] Proteomic approaches have emerged as an invaluable tool to understand the sperm physiology and pathogenic mechanisms associated with male infertility, and also comparing the abundance of thousands of proteins simultaneously in different subtypes of infertile patients might facilitate the identification of fertility biomarkers useful for the clinics or for the design of new fertility therapies or male contraceptive targeting.

Potential Clinical Use

Comparative semen proteomic studies from various functional states have produced a large number of candidate fertility biomarkers. A reliable biomarker should be accessible using noninvasive protocols, inexpensive to quantify, with a detection method that is sensitive and specific as well as highly reproducible among clinical laboratories. Although the extreme value of high-throughput proteomics as a biomarker discovery tool has been proven, some limitations hamper its routine use in the clinics.[46] First of all, sperm proteomics biomarker discovery experiments have shown so far a relatively low concordance between different laboratories. This is exemplified by the detection of only 17 out of the 179 differentially expressed proteins in at least two of the seven comparative studies in asthenozoospermia (Figure 18.3b). Interestingly, heat shock-related 70-kDa protein 2 (HSPA2) was found differentially expressed in four of the seven studies assessing protein changes in asthenozoospermia, suggesting that HSPA2 might be a good biomarker for altered sperm motility. The causes of the lack of detection of the same proteins between studies may be due to the following reasons: differences in sample collection, handling, and storage; different proteomic technologies applied; proportion of the proteome targeted; and the biological intra- and interindividual variance. Thus, because of the wide variety of conditions and approaches, the results obtained so far must be interpreted as being complementary rather than indicating genuine lack of concordance or reproducibility. Another limitation of the use of proteomics in the clinics is its prohibitive associated cost, including the requirement of skilled professionals and very expensive equipment. Thus, despite the latest advances in mass spectrometry technology, semen proteomics has only been used as a research and biomarker discovery tool so far. However, once the clinical value for fertility/infertility for some of the candidate biomarkers detected by proteomics is validated, it will be possible to develop cheaper and more feasible tests such as those based on protein microarrays, mass spectrometry selective reaction monitoring (SRM), or ELISA multiplexed to routinely test these specific biomarkers in the reproductive clinics. The results derived from the study of the semen proteome might provide an enhancement in some reproductive clinical applications as it is presented below.

Improvement in the Reproductive Counseling

Fertility treatment options can range from basic advice from the doctor to the most sophisticated therapies such as in vitro fertilization (IVF). The less invasive treatments include time intercourse (TIC), which simply identifies the days of the menstrual cycle when the woman could become pregnant, and IUI, which is based on the injection of sperm inside a woman's uterus, thus increasing the number of sperm that reach and could fertilize the oocyte. In comparison, IVF combines a sperm and an oocyte outside of the body in a laboratory dish with or without the help of ICSI, and only one or two fertilized eggs that start to develop to embryo are transferred into the woman's uterus. Reproductive counseling of infertile couples initiates with an extensive physical and molecular evaluation of the female and a basic physical analysis and seminal parameters evaluation in males.[47] Each couple receives advice about the appropriate fertility treatment based on evidence-based information about the success rate of different treatment options depending largely on the cause of infertility and the associated costs. Furthermore, the good practice in the reproductive clinic should also ensure that patients are not exposed to unnecessary invasive technologies or ineffective treatments. For example, when a known severe male or female infertility factor is identified (e.g., an ovulatory or tubular disorder in females or the diagnosis of azoospermia or severe oligoasthenozoospermia in males), the patients are advised to consider in vitro ART as the first treatment option. In contrast, reproductive treatments with minimal intervention are the first treatments that should be suggested for infertile couples with unexplained infertility or with mild to moderate female or male factor,[48] therefore reducing the clinical exposure of the women to intense treatments such as ovary hyperstimulation and egg collection. Although in vitro ART has a high success rate for couples with severely compromised semen parameters (around 40%), the success of TIC or IUI in infertile patients without severe alteration of seminal parameters is unpredictable. Only a single study attempted to identify potential protein biomarkers able to predict pregnancy outcome

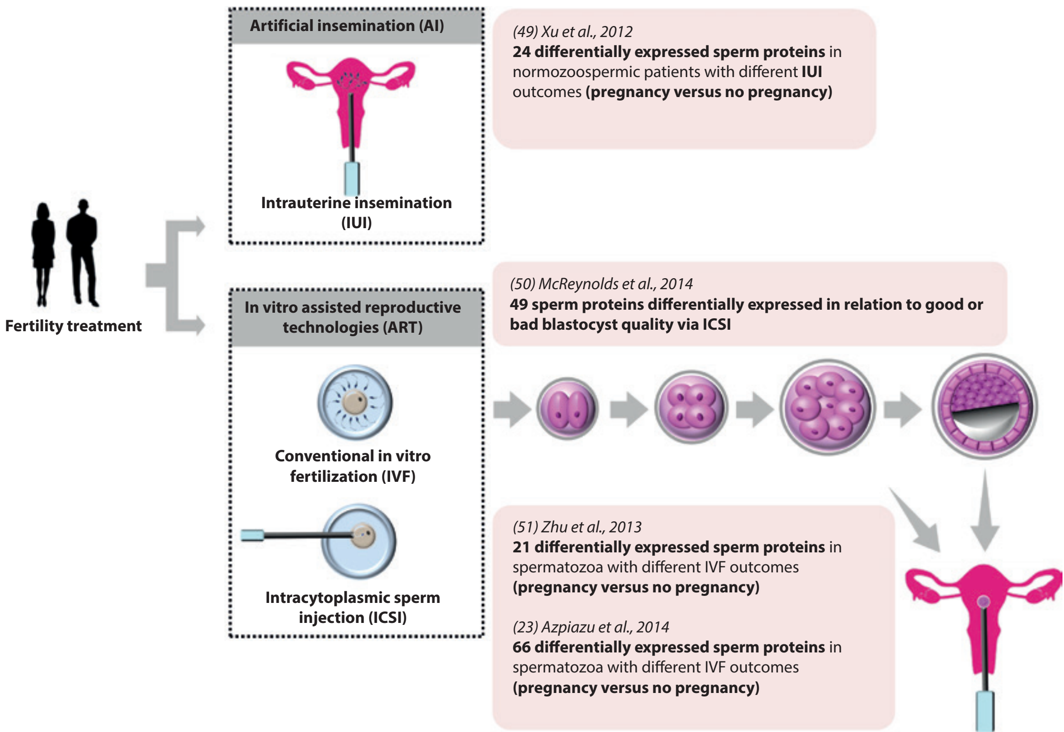

FIGURE 18.4 Comparative sperm proteomics related to assisted reproductive technology (ART). Comparative proteomic studies related to different ART outcomes (intrauterine insemination [IUI], in vitro fertilization [IVF], intracytoplasmic sperm injection [ICSI]) have detected several differentially expressed sperm proteins potentially able to predict success (pregnancy or blastocyst quality) from different fertility treatments.

by IUI (Figure 18.4).[49] A total of 24 differentially expressed proteins involved in sperm capacitation, acrosomal reaction, and sperm oocyte communication were identified in patients with normal semen parameters but unable to achieve a live birth by IUI.[49] Additionally, to enhance the outcome by in vitro ART, three different studies assessed the proteomic changes in sperm in relation to blastocyst quality development[50] and final pregnancy outcome[23,51] after in vitro ART. A total of 136 differential proteins were detected by the three different studies but only seven proteins were commonly affected in at least two of the three studies (Semenogelin-1, Semenogelin-2, Clusterin, Peroxiredoxin-5 mitochondrial, 5-oxoprolinase, Cysteine-rich secretory protein 2, and uncharacterized protein C17orf74). The low reproducibility between the few studies assessing the proteomic changes associated to the in vitro ART success indicates that we are in the infancy of the proteomics era in reproductive clinics. Infertility is a complex disease with underlying multiple causes. Therefore, an unmet need is the development of a panel of molecular biomarkers able to discern the male factor infertility and, thereby, be predictive of the different fertility treatment success reducing emotional and economical burn of the couples facing reproductive care.

Improvement in the Counseling of Azoospermic Patients Facing Invasive Procedures

Azoospermia, defined as the complete lack of spermatozoa in the ejaculate, is a severe disorder affecting nearly 5%–20% of infertile men.[22,52,53] Azoospermia is mainly classified as obstructive azoospermia (OA) or as nonobstructive azoospermia (NOA).[54] The majority of the patients with OA exhibit normal spermatogenesis and spermatozoa could be recovered for in vitro ART purposes by testicular sperm extraction (TESE).[55] In contrast, this invasive procedure (TESE) is not recommended for patients with NOA presenting a complete lack of spermatogenesis as, for example, for patients diagnosed as Sertoli cell-only syndrome (SCOS).[22] However, if NOA patients presented hypospermatogenesis or maturation arrest, there is the possibility to retrieve live spermatozoa from testis biopsy fragments, although with a low efficiency.

Currently, the main diagnostic method to discern OA from NOA and its different subtypes (hypospermatogenesis, maturation arrest, and SCOS) is the testicular biopsy.[56] There is a particular interest to explore whether some specific protein biomarkers in semen could be predictive for the presence of sperm in testis. The identification of such potential spermatogenic predictive biomarkers could let to the development of tests to avoid that patients without possibilities to recover spermatozoa (e.g., patients diagnosed as SCOS) underwent invasive and painful procedures such as testicular biopsy. Additionally, the diagnosis of NOA by testicular biopsy is not very accurate because it does not reflect the histology of the whole testis. Even if the general spermatogenesis within seminiferous tubules is not progressing in NOA patients, occasionally in some tubules sperm cells could be detected.

Proteomics has revealed several differential expressed proteins in the seminal plasma of men with different subtypes of azoospermia compared with individuals with normal spermatogenesis.[22,54,55,57–59] Recently, using MS/MS followed with a selected reaction monitoring (SRM), two protein biomarker candidates (epididymis-expressed protein ECM1 and the testis-expressed protein TEX101) were proposed for differential diagnosis of azoospermia (Figure 18.5).[60] The authors suggest that these two proteins are capable of differentiating OA from NOA as well as the different NOA subtypes. The high level of ECM1 expression in epididymis enables discerning patients with OA showing lower ECM1 expression than individuals without obstructive disorders including individuals with normal spermatogenesis or NOA patients.[60] Additionally, if the germ-specific protein TEX101 is almost absent in the seminal plasma, this is suggestive of absent spermatogenesis (SCOS) or vas deferens obstruction (OA and postvasectomy patients). In contrast, TEX101 is detected in higher levels in patients presenting maturation arrest or hypospermatogenesis. The potential future use of these two biomarkers (ECM1 and TEX101) in the reproductive clinics has the potentiality to avoid testicular biopsy for TESE retrieval in cases of pure SCOS as well as to improve the NOA differential diagnosis, thus reducing the cost of azoospermia counseling. Other authors have proposed other proteins such as clusterin (CLU), prolactin-inducible protein (PIP), galectin-3-binding protein (LGALS3BP), L-lactate dehydrogenase C chain (LDHC), phosphoglycerate kinase 2 (PGK2), and transketolase-like protein 1 (TKTL1) as complementary spermatogenic biomarkers.[54,59]

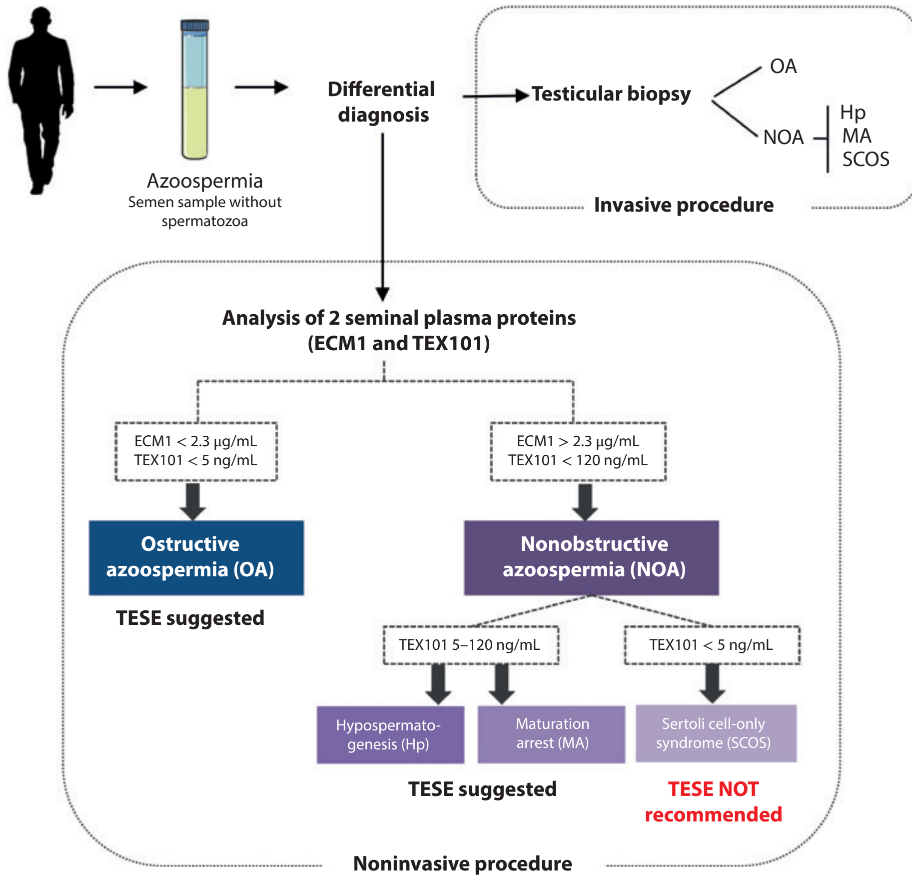

FIGURE 18.5 Semen protein biomarkers in the differential diagnosis of azoospermia. Testicular biopsy is the current main diagnostic methodology to diagnose obstructive azoospermia (OA) versus nonobstructive azoospermia (NOA) and its subtypes: hypospermatogenesis (Hp), maturation arrest (MA), and Sertoli cell-only syndrome (SCOS). Also, molecular tests such as karyotyping, Y-chromosome microdeletion studies or CFTR testing, and physical examination and ultrasounds are useful in some but not all azoospermic cases to assist in the differential diagnosis of azoospermia. A new diagnostic tool using two semen proteome biomarkers (epididymis-expressed protein ECM1 and the testis-expressed protein TEX101) is able to discern the different subtypes of azoospermia. Seminal plasma levels of ECM1 protein <2.3 µg/mL suggest an epididymal or vas deferens blockage, whereas values >2.3 µg/mL are suggestive of NOA. Low seminal plasma levels of germ-cell protein TEX101 <5 ng/mL indicate azoospermia due to lack of spermatogenesis for the presence of SCOS or OA. On the other hand, NOA men with affected spermatogenesis (Hp and MA) have TEX101 seminal levels between 5 and 120 ng/mL. NOA patients presenting >5 ng/mL of TEX101 protein in seminal plasma suggest the presence of spermatogenesis, and sperm retrieval by testicular sperm extraction (TESE) is allowed. In cases of SCOS (<5 ng/mL), TESE should be avoided.[60]

Improvement of Clinical Processes

Cryopreservation of human sperm is widely applied in the field of reproductive biology and medicine. Sperm cryopreservation has enabled preservation of male fertility particularly for those men who will undergo potentially sterilizing anticancer treatments. Furthermore, cryopreservation allows the creation of sperm donor banks including the storage of sperm recovered by TESE for future uses, thus avoiding repetitive biopsies in azoospermic patients. An initial comparative proteomic study of fresh and cryopreservated sperm detected a total of 27 differentially expressed sperm proteins.[61] Protein degradation and alteration of posttranslational modifications such as phosphorylation were proposed as the potential cryoinjury mechanisms involved. Proteomic studies may help design new cryogenic strategies to improve the sperm protection against freezing. In a more recent study, the application of TMT

technology coupled to LC-MS/MS led to the detection of substantial changes in the sperm proteome at every stage of the cryopreservation process, including the effect of the cryoprotectant itself, which may ultimately impair the sperm fertilizing capability.[72]

Design of Potential Fertility Therapies

Proteomics might help identify the key proteins for male fertility. New fertility enhancers might be developed based on these key proteins. Toward this objective it has been described that around 20%–40% of infertile males present high levels of oxidative stress[62] and antioxidant intake therapy is showing beneficial effects on these infertile males. However, several types of antioxidant therapies exist and the optimal type and dose have not been established yet.[63] Proteomic studies comparing infertile males presenting different levels of reactive oxygen species (ROS) with fertile males have resulted in the identification of several proteins involved in the oxidative stress.[64–66] These differential proteins might help standardize the antioxidant therapies as well as predict those patients who are going to positively respond to the therapy.

Design of Potential Anticonceptive Strategies

Proteomics may also help identify new targets for male contraception. Hormonal methods such as the administration of exogenous testosterone have shown the partial or total suppression of spermatogenesis resulting in oligozoospermia or azoospermia, respectively. Although hormonal anticonceptive methods are reversible they have several side effects. Proteomic analysis of human testicular biopsies in men before and after exogenous testosterone treatment resulted in the detection of 13 differential expressed proteins. Those differential proteins, probably crucial for a normal spermatogenesis, might be good candidates for new potential reversible male contraception methodology, although their clinical use should be elucidated.[67] Using a different approach proteomics has also been applied to the study of sperm immunogenic antigens, both with a view to understand immunologic infertility and also to identify potential immunocontraceptive candidates.[68–70]

Test Availability

A recent patent application on the use of identified germ cell-specific proteins in an antibody-based assay (Fertichip™) to predict the successful testicular biopsy outcomes in human nonobstructive azoospermia is being developed based on a combination of different proteins, although the test is not yet available in the market.[59] The SpermCHECK® Male Fertility Test is an example of an application based on the detection of a single protein. It works by detecting the concentrations of the acrosomal protein SP-10 (ACRV1), known to be present in the sperm head cell membrane, to determine sperm count number or presence.[71] This is an at-home sperm test and is already available on the market (http://www.spermcheck.com/) through different retailers in the United States, Canada, UK, Hong Kong, Macau, and France. As the field of sperm cell proteomics further advances it can be expected that many applications will become available based on the detection of single proteins or on the combination of proteins.

Acknowledgments

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19

Evaluation of Surgically Retrieved Spermatozoa and Its Usefulness in Assisted Reproduction

Biljana Popovic-Todorovic, Greta Verheyen, Francisco Osorio, and Herman Tournaye

Introduction

Male infertility treatment has been revolutionalized twice: the first time by the introduction of intracytoplasmatic sperm injection (ICSI) in 1992[1] and the second time when ICSI allowed spermatozoa retrieved from the testis to fertilize an oocyte leading to viable embryos and healthy pregnancies.[2,3]

Azoospermia is defined as the absence of spermatozoa in the ejaculate after assessment of centrifuged semen on at least two occasions. It is observed in 1% of the general population and in 10%–15% of infertile men.[4] It can be clinically classified as either obstructive (OA) or nonobstructive azoospermia (NOA) of which the latter constitutes 60%.[4,5]

Epididymal sperm aspiration techniques (microsurgical epididymal sperm aspiration [MESA], percutaneous epididymal sperm aspiration [PESA]) and testicular sperm extraction techniques (fine needle aspiration [FNA]/testicular sperm aspiration [TESA], testicular sperm extraction [TESE], microsurgical testicular sperm extraction [mTESE]) have now become the predominant tools to obtain sperm suitable for assisted reproductive technology (ART) in azoospermic patients.[6]

A Cochrane meta-analysis on surgical sperm retrieval techniques concludes that there is insufficient evidence to recommend any specific sperm retrieval technique and that the least invasive technique should be used.[7]

Currently, surgical techniques are reaching a plateau in terms of sperm retrieval rates, with varying success rates according to the specific diagnosis or selection of the patients. Sperm can be retrieved in virtually all cases of OA, but only in around 50% of NOA when no preliminary selection of patients on the basis of histopathology has been performed.[8] Furthermore, men with NOA have lower fertilization and clinical pregnancy rates compared with men with obstructive azoospermia having normal spermatogenesis.[9] Therefore, the contribution of the ART laboratory has become increasingly important, specifically in developing methods to enhance the retrieval of spermatozoa from the tissue, to avoid sperm damage and to improve the selection of good quality sperm for ICSI.

Sperm Processing and Selection

The identification and preparation of spermatozoa for fertilization are key steps of in vitro fertilization (IVF)/ICSI procedure. Sperm selection strategies can significantly impact the quality of the spermatozoa used for ART and strongly influence the reproductive outcomes. Some investigators suggest that the principles of sperm selection in vitro should mimic the natural selection process that occurs in the female reproductive tract.[10,11] Traditional techniques are based on motility and morphology and do not take into account functional aspects or genetic quality of the sperm.

Methods for Processing Epididymal and Testicular Sperm Samples

Recovery of Epididymal Sperm

Since obstructive azoospermia is the indication for epididymal aspiration, it is possible to obtain large numbers of spermatozoa with minimal contamination by red blood cells and nongerm cells. The aspirates are emptied into a tube containing a buffered medium containing heparin. After mixing with an automatic pipette, a drop is evaluated in a Makler or Neubauer counting chamber.

If sufficient numbers of epididymal sperm cells are collected, density gradient centrifugation can be used to prepare the spermatozoa for ART. On the other hand, the simple wash technique will be used if the aspirated number of spermatozoa is low.[12]

Recovery of Testicular Sperm

Testicular samples contain large numbers of different cell types and debris, especially red blood cells. Additionally, the elongated spermatids, which are still attached within the seminiferous tubules, must be freed. A number of methods can be used to isolate the spermatozoa.

Mechanical Methods

Shredding Method

This procedure is used to prepare testicular biopsy samples as the sperm are contained within the seminiferous tubules. The testicular tissue is excised by the surgeon (Figure 19.1). The tissue is placed in a Petri dish with HEPES-buffered medium and then finely minced and teased apart with fine needles (Figure 19.2). Many of the sperm found are immature or immotile; some are motile, often with a large cytoplasmatic droplet attached to the neck. The minced tissue is placed in a 5 mL Falcon tube and centrifuged for 5 minutes at 1800 × *g*. After this the pellet is resuspended in 0.2 mL of culture media.[13] Then the embryologist can search for suitable, mature-looking motile sperm for ICSI (Figure 19.3).

Squeezing Method

Seminiferous tubules are teased apart and rinsed to remove blood contamination, and they are subsequently placed in a Petri dish with fresh culture media. Tubules are then cut into short lengths (1–2 cm) with fine needles. A long, thin Pasteur pipette is pulled over a flame and then bent (ideally at an angle of 45°). A second pipette (without a bend) should be heated, pulled, and used to pick up the tubule contents. By holding one end of a cut tubule with the point of a needle, the bent pipette can run along the length of the

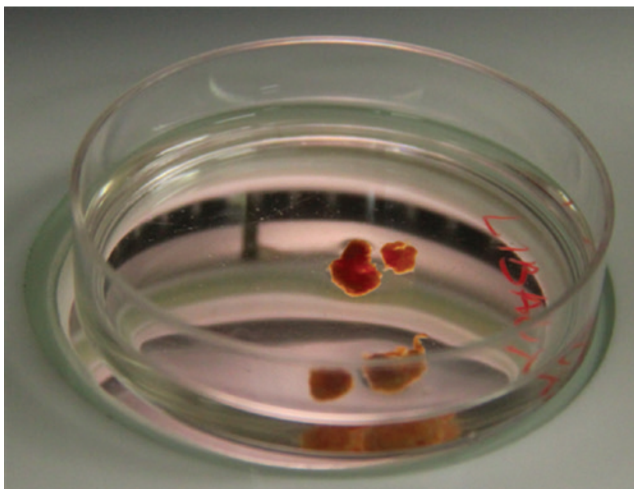

FIGURE 19.1 Surgically retrieved testicular tissue.

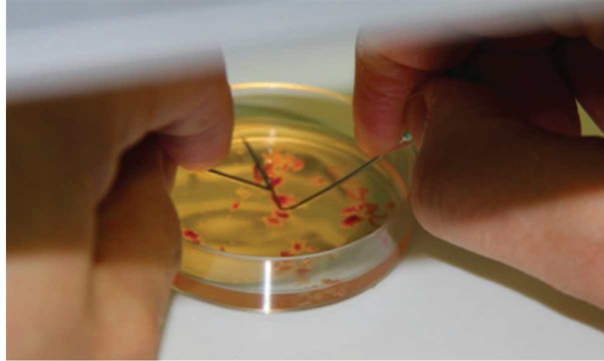

FIGURE 19.2 Mechanical shredding with needles of a testicular sample.

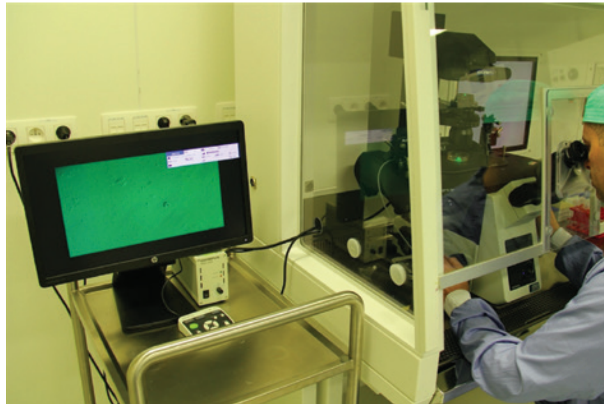

FIGURE 19.3 Searching for the presence of sperm in the wet preparation under ×400 magnification.

tubule by simultaneously pushing down against the base of the Petri dish. This procedure squeezes the entire contents of the tubule into the medium. The contents can now be picked up with the second pulled pipette and placed in a test tube filled with clean sperm media or placed on a slide to look for sperm.[14]

Cell Strainer

A cell strainer is used to remove unwanted debris when processing large biopsy samples. After teasing apart and slicing the biopsy sample with fine needles, the slices are rinsed in a series of Petri dishes with sperm preparation media to remove any blood contamination. The tubules are then placed in a cell strainer (Becton Dickinson & Company, Franklin Lakes, New Jersey). Heat treating the end of a clean, sterile Pasteur pipette produces a sphere-shaped tip approximately 5 mm in diameter. This pipette is used as a pestle to grind and break up the seminiferous tubules against a mesh strainer.[14]

Tissue Grinder Method

A mini tissue grinder can be used to process large tissue samples.[15] First, the tissue is teased apart, then sliced with fine needles and placed in a test tube with fresh culture media and a glass pestle. With the glass pestle, the sample is ground at the bottom of a glass tube. After concentrating the sample by centrifugation, the pellet is resuspended with fresh culture media and placed on a Petri dish in 10 μL drops to look for sperm.[15]

Erythrocyte-Lysing Buffer Method

The erythrocyte-lysing buffer (ELB) method is applied when the biopsy sample is highly contaminated with blood. Nagy et al. first used the ELB to lyse the red blood cells in the tissue pellet.[16] The presence

of high concentrations of erythrocytes and few sperm in NOA can make the examination of the sample a very lengthy process. Sperm visualization improves substantially after lysing the erythrocytes.

Shredded biopsy specimens are suspended in Hepes-buffered medium and centrifuged for 5 minutes at 300 × *g*. Testicular sperm pellet is resuspended in 2–4 mL of erythrocyte-lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 2 mM ethylenediaminetetraacetic acid; pH 7.2) for 10 minutes at room temperature. Then 5–10 mL of Hepes-buffered medium supplemented with protein is added to the suspension and the tube is centrifuged for 10 minutes at 500 × *g*. The pellet is then resuspended with 1.5 mL of culture medium with protein. This resuspended pellet can be transferred to an Eppendorf tube, washed, and centrifuged again at 500 × *g* for 5 minutes. After centrifugation, the pellet is resuspended in 50 µL of culture medium supplemented with protein. Five to 10 µL droplets of this pellet is placed in a dish. Subsequently, the embryologist can search for spermatozoa in these droplets.

This technique enhances the efficiency of sperm collection, providing comparable fertilization and embryo development rates. In a series of NOA patients with no sperm recovery after a 1 hour search in the shredded biopsy suspension, in 14 out of 41 (34%) men it was possible to recover spermatozoa in the cell suspension treated with ELB.[17]

Enzymatic Digestion Method

In cases when the sperm production is limited, especially in patients with NOA, extraction of large amounts of testicular tissue is necessary to increase the chance of finding the foci of spermatozoa. The large tissue volumes that need to be processed in combination with very small sperm numbers makes mechanical extraction and search for spermatozoa very complicated and time consuming. The use of enzymes to digest the surrounding testicular tissue that sometimes hides the sperm is a very good tool to improve sperm recovery. The first report[18] used collagenase type IA and DNAse, but the minimum incubation time was 4 hours resulting in an important decrease of sperm motility. Also collagenase type IV has been found to be efficient for testicular sperm recovery.[19]

The most widely used protocol consists in incubating the tissue suspension in a solution containing 1000 IU/mL of collagenase type IV and 25 µg/mL of DNAse at 37°C for 1 hour.[19]

Biopsy specimen is firstly minced with fine needles or forceps in a Petri dish until tissue pieces of ~1 mm^3 or free tubule pieces of a few millimeters in length are obtained. The suspension is placed in a tube (Figure 19.4). After sedimentation of the remaining tissue pieces, the supernatant is centrifuged at

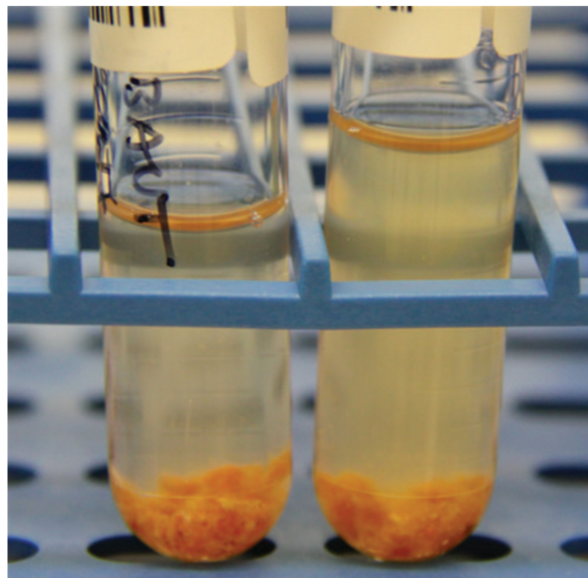

FIGURE 19.4 If the wet preparation does not show any spermatozoa, the tissue is placed in tubes with buffered medium and centrifuged.

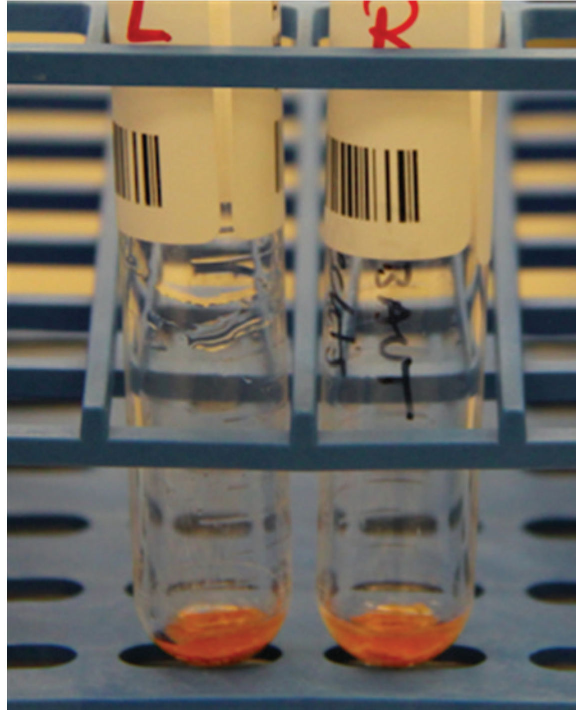

FIGURE 19.5 After centrifugation, before addition of collagenase type IV (GM501 Collagenase® Gynemed, ready to use).

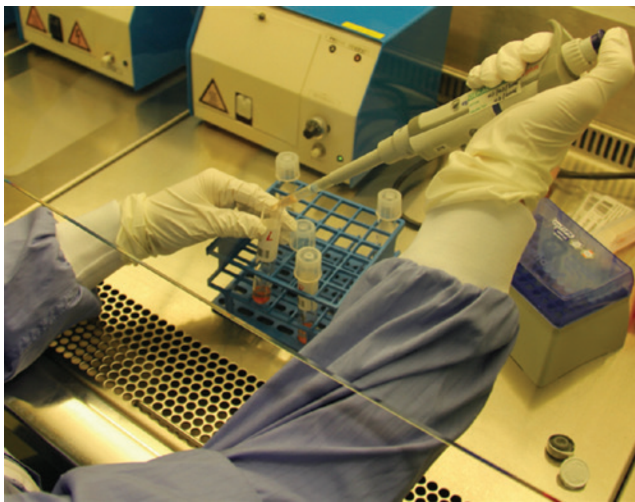

FIGURE 19.6 Addition of collagenase type IV to the tubes (GM501 Collagenase® Gynemed, ready to use).

300 × *g* for 5 minutes (Figure 19.5) and spermatozoa are directly recovered from this resulting pellet. elb may be used to increase the probability of visualizing any spermatozoa or elongated spermatids in the case of high concentrations of red blood cells. If no sperm is found, the residual tissue pieces are placed in 1 mL of Hepes-buffered medium supplemented with protein, 1.6 mM $CaCl_2$, 25 μL/mL DNase, and 1000 IU/mL collagenase Type IV (Figure 19.6). The tissue samples are then placed in an incubator at 37°C for 1 hour to allow digestion to occur (Figure 19.7). To facilitate complete enzymatic digestion, the samples are shaken every 10–15 minutes during the incubation period. The digested tissue solution

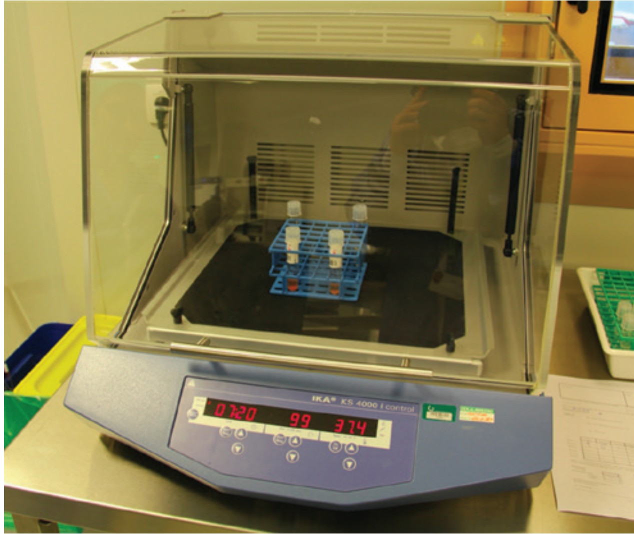

FIGURE 19.7 Incubation of the sample for 1 hour under constant shaking during the enzymatic digestion.

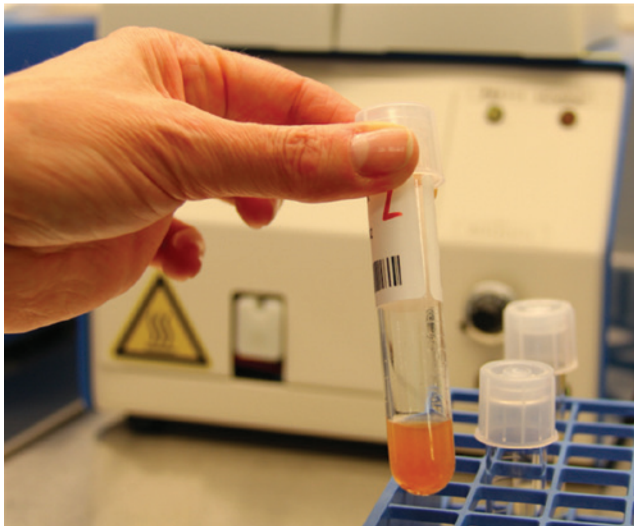

FIGURE 19.8 Cell suspension following enzymatic digestion and incubation.

is gently centrifuged for 5 minutes at 50 × *g* to remove any residual pieces or debris not dissolved by the enzymes (Figure 19.8). The remaining cell suspension (supernatant) containing loose cells is then washed twice with Hepes-buffered medium (Figure 19.9) and centrifuged for 5 minutes at 1000 × *g*. The pellet is resuspended and multiple small droplets of 5 μL are placed in a Petri dish and covered by oil to search for spermatozoa under the inverted microscope and retrieve spermatozoa for ICSI.[19] At our center, the droplet from the pellet is placed on a glass slide and examined under the microscope at ×200–400 magnification (Figures 19.10 and 19.11).

This method can be considered successful in reducing sperm recovery failure and may increase the chance of selecting the highest quality spermatozoa in patients with NOA. In a series of Crabbé et al.,[17] it was impossible to obtain spermatozoa after mechanical mincing and the use of erythrocyte lysing buffer in 27 out of 41 patients. In 7 out of these 27 patients (26%) spermatozoa for ICSI were retrieved after enzymatic digestion.[17]

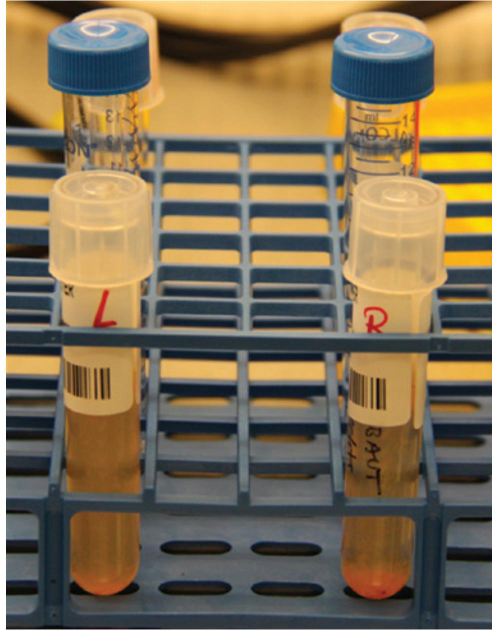

FIGURE 19.9 Cell suspension containing loose cells is washed twice and centrifuged.

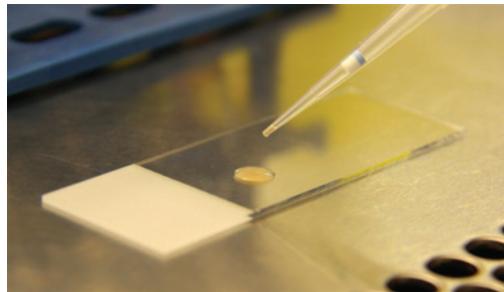

FIGURE 19.10 Droplet of the pellet is placed on a glass slide.

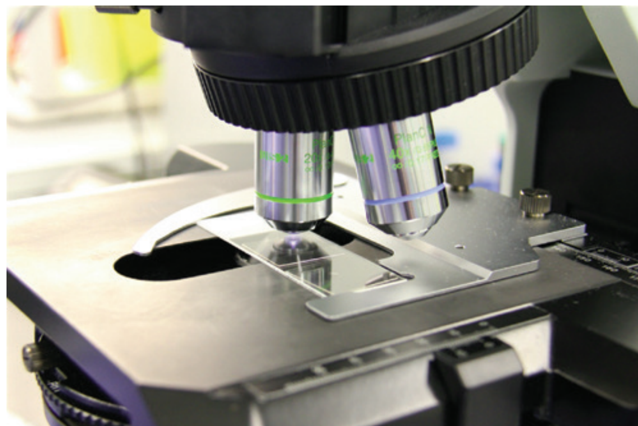

FIGURE 19.11 Searching for sperm under the microscope at ×200–400 magnification.

Sperm Selection Methods

Vital spermatozoa are a prerequisite for successful ICSI. For the ART laboratory, the primary means of determining a spermatozoon's vitality is by its ability to move.

Complete asthenozoospermia can either be caused by a structural inability of spermatozoa to move (e.g., Kartagener's syndrome) or it can be due to infection or numerous other influences.[20] On the other hand, immotility in testicular samples is a rather normal event. Still motility can be observed in OA but also in NOA.

There are authors who advise the sperm retrieval to be done a day prior to oocyte retrieval since the motility improves in culture.[21,22] However, other groups did not find any effectiveness of in vitro culture and on sperm motility.[23]

Testicular sperm should not be cultured for more than 48 hours to increase motility due to the aging of spermatozoa. An increase in structural chromosomal abnormalities and DNA fragmentation in in vitro-stored spermatozoa has been reported.[24]

The most commonly used sperm selection methods include the sperm tail flexibility test (STFT), the hyposomotic swelling test (HOS) test, the use of chemical substances for induction of tail movements, and laser-assisted immotile sperm selection (LAISS).

STFT/Mechanical Touch Technique

STFT/mechanical touch technique is based on the observation that the immotile vital spermatozoa have a flexible tail and immotile nonvital spermatozoa have a rigid tail. The technique involves lateral touching of the immotile sperm tail with the ICSI micropipette, forcing the tail to one side. If the tail is flexible and recovers its original position, the spermatozoon is considered viable. Tail rigidity and incapacity to recover the initial tail position are considered a sign of nonviability; in other words, if the head moves together with the tail then the spermatozoon is unsuitable for ICSI. The success and reliability of this technique depend largely on the expertise of the biologist performing the assessment. Although this technique has been applied since the early years of ICSI, it was first described by de Oliveira in 2004.[25]

The use of this technique on both frozen and fresh TESE-ICSI cycles has been found to result in similar fertilization rates whether immotile or motile sperm were injected (65.7% vs. 74.3% frozen, 73.4% vs. 64.4% fresh, respectively). There were no differences in pregnancy and delivery rates in both groups, indicating that the selection method was able to identify viable but immotile spermatozoa.[25]

This method may not be the best for all cryopreserved and thawed ejaculated samples because of spontaneous tail curling, which could have an influence on the rigidity of the tail (making them indistinguishable from those which are not vital).[15]

This method is advantageous in relation to other techniques since it does not change the structural integrity of spermatozoa, there are no additives used, and no cleaning is required, meaning that the spermatozoa can immediately be injected. There are no concerns over possible detrimental effects on the embryo because no additives are used.

It must be stated that STFT is not 100% accurate and requires a high level of expertise of laboratory personnel. Moreover, in cases with very few sperm, it may be very time consuming to touch them one by one.

The Hypoosmotic Swelling Test

The HOS was developed by Jeyendran et al. to evaluate the functional integrity of the sperm membrane.[26] The modified HOS test using culture medium and deionized grade water (1:1) for selecting immotile sperm for ICSI was introduced by Verheyen et al.[27] Viable sperm with normal membrane function will exhibit tail swelling and curling due to the influx of water when exposed to hypo-osmotic conditions. After exposure for maximum 10 seconds and identification of these spermatozoa, they can be retrieved and placed in a normo-osmotic fluid where they regain normal shape prior to injection.[28] Various groups have used the HOS test and modifications have been developed.[29–32]

The HOS test improves fertilization rates not only in fresh TESE spermatozoa but also in frozen–thawed samples. Fertilization rates increased from 30.3% to 44.0% in the fresh and from 25.7% to 42.7% in the frozen TESE group, just by HOS test selection.[31]

Because the HOS test depends, in part, on the sperm tail membrane, it is not very useful when there are anatomical sperm tail deficiencies, functional sperm–tail and flagellar defects.[33]

Chemical Motility Enhancers

Pentoxyfylline is a phosphodiesterase inhibitor of the methylxanthine group. It inhibits the breakdown of cyclic adenosine monophosphate (cAMP), a molecule known to play a role in sperm motility.[34] It was found that adding pentoxyfylline to a testicular sperm sample caused immotile sperm to become motile. This procedure is performed by adding pentoxyfylline to the sperm suspension at a 1:1 ratio so that the final concentration of pentoxyfylline in the sample is 0.5 mg/mL. Following the incubation at 37°C for 20 minutes, the sample is observed for the identification and isolation of motile sperm.

Addition of pentoxyfylline significantly improved fertilization rates and increased the number of available embryos compared with cycles where no artificial activation was used and selection was performed only according to the rigidity of the tail, but there were no differences in pregnancy rates.[35] When testicular spermatozoa were treated with pentoxyfylline, significantly higher motility was observed.[36] Irrespective of heterogeneous data regarding clinical outcome, the use of pentoxyfylline significantly reduces the time spent in the laboratory in finding and selecting motile spermatozoa.[36]

Another member of the xanthine family, theophylline, has recently been evaluated in a prospective trial on sibling oocytes. It was shown that it has an immediate but short-term effect on sperm motility.[37] A ready-to-use product is commercially available (GM501 SpermMobil® Gynemed).

There have been concerns regarding the safety of these compounds as the data come from animal studies.[38] There was no evidence of abnormalities in offspring where live births were reported in the clinical setting following pentoxyfylline or thepphylline treatment.

Laser-Assisted Immotile Sperm Selection

Aktan et al. developed a technique with use of the laser for the identification of viable but immotile spermatozoa.[39] A single laser shot of 129 μJ for approximately 1.2 milliseconds is directed to the tip of the flagellum that in a live but immotile spermatozoa causes a curling or coiling of the tail. Conversely, if no such change is seen then the spermatozoon is nonviable. The number of viable spermatozoa in a testicular sample identified by LAISS was comparable with that of the HOS test (22.0% vs. 21.5%).[39] The fertilization rate improved in the laser selection group, from 20.4% in the randomly selected TESE spermatozoa group to 45.4%; accordingly, the take-home baby rate increased from 5.9% to 19.0%.[39]

The advantage of laser use is that it does not require chemical compounds to induce motility, so it has no side effects. It requires skilled personnel to be routinely used. The main obstacle to its widespread application remains the cost of the instrument.[40]

Cryopreservation of Surgically Retrieved Testicular Sperm

Cryopreservation of human spermatozoa is a routine procedure in assisted reproductive technology. Cryopreservation of testicular spermatozoa can avoid repeated testicular biopsies.[41] Repeated testicular surgeries can cause permanent testicular damage, irreversible atrophy, deterioration of spermatogenic development, and possibly a loss of endocrine function. As the success of repeated retrievals is not warranted,[9] cryopreservation, especially in men with nonobstructive azoospermia, to some extent ensures the availability of sperm at the time of ICSI.

The issues with the cryopreservation of testicular sperm arise from the fact that the number of spermatozoa is low and motility is poor.[42] Given the unique characteristics of epididymal and testicular spermatozoa, conventional methods of sperm cryopreservation may not be optimal. Several different methods

for the cryopreservation of testicular and epididymal sperm are available. The choice of the container or carrier may be an important consideration and should take into account the number or concentration of the sperm in the final preparation.

In 1999, Crabbé et al.[43] showed that freezing of testicular sperm in a suspension preserved sperm quality better than whole-biopsy freezing, with glycerol being used as a cryoprotectant.[43] As for ejaculated sperm, glycerol is also the cryoprotectant of choice also for testicular sperm. Freezing media, containing glycerol and extenders to increase cryosurvival, are commercially available from several companies. The applied cryopreservation procedures for testicular sperm (vapor or programmed freezing) are largely comparable to those of ejaculated sperm, as the mature spermatozoa are the important cells to be preserved.

The most widely used carrier, even in the case of small numbers of sperm, is the closed high-security straw, composed of an ionomeric resin (CBS, CryoBioSystem, Paris, France). Different carriers have been investigated when the number of sperm in a testicular biopsy sample is extremely low. Cohen et al.[44] described a method where cryopreservation and recovery of spermatozoa can be performed even in patients who have fewer than 100 spermatozoa present in the final testicular tissue homogenate.[44] A porous capsule, such as an emptied zona pellucida, is used as a vessel to contain individual spermatozoa. The use of an empty zona pellucida showed advantages in cryopreservation of low numbers of spermatozoa and also reduced the loss of motility associated with postthaw dilution and sperm washing, which is observed when thawing frozen donor sperm.[44] Emptied zona pellucida from rodents (mouse or hamster)[44–46] as well as from humans[44,45,47] have been used. The use of zona pellucida of heterologous and nonhuman origin, however, has raised ethical concerns, and the technique has never been widely applied due to its complexity.

Various types of containers have been used in an attempt to freeze small number of spermatozoa such as droplets on plastic dishes,[48] ministraws,[49] micropipettes,[50] cryoloops,[51,52] copper loops,[53] Volvox globator algae,[54] agarose microspheres,[55] and alginate beads.[56]

Each of the methods is associated with a number of disadvantages and as such has not gained widespread use. All of the methods are discussed in detail in a review by Abdel Hafez et al.[42]

Vitrification as a method of cryopreservation has significantly improved the outcome of infertility treatment regarding cryopreservation of oocytes and embryos. An advantage of vitrification, as an alternative to conventional cryopreservation, is that no cryoprotectants are needed, thus avoiding the lethal effects of cryoprotectant toxicity and osmotic damage specifically to spermatozoa.[53] Vitrification of human sperm has only been considered since 2002 when Nawroth et al.[57] developed a new vitrification method for human sperm involving rapid nonequilibrium freezing and thawing and avoiding the use of high concentrations of permeable cryoprotectants.[57] They reported the use of nonpenetrating sucrose in concentrations of 100–250 mM.[57]

Studies are emerging on vitrification of a small number of sperm. Endo et al. have investigated the use of vitrification containers CryoTop (open system) and as few as five spermatozoa in a Cell Sleeper (closed system) on discarded specimens after IVF/ICSI procedures.[58,59] Kuzneytsov et al. have shown good vitrification postthaw recovery of a small number of spermatozoa using only nonpermeating cryoprotectants in a closed straw system in normo-zoospermic and severely oligozoospermic samples.[60] Based on animal studies, Gianaroli et al.[61] performed freeze drying or lyophilization of human sperm. Although the reproductive potential of freeze-dried sperm after ICSI has been shown in the cow, rehydration of human sperm resulted in total loss of motility and viability.

Regardless of the container or the cryopreservation method used, no prospective, randomized controlled trials were performed to show that any single carrier or method is superior to the others. Novel cryopreservation technology specifically designed to handle small numbers and quantities of sperm need to be further explored. The current evidence is not sufficient to support the use of one technology over the other. Well-designed clinical trials with appropriate sample sizes are needed to assess the feasibility and efficiency of various low sperm count freezing methodologies.[42]

The Use of Surgically Retrieved Sperm in Assisted Reproduction

Despite the fact that surgically retrieved sperm has been used for more than 20 years, a number of issues remain regarding ART use.

In patients with obstructive azoospermia, the spermatogenesis is mostly not affected and the treatment prognosis is good. Obstruction can be acquired (vasectomy, infection, or trauma) or congenital. The most common is congenital absence of the vas deferens (CBAVD), which is linked to the mutations in the cystic fibrosis transmembrane-conductance regulator gene. The current evidence states that the surgical retrieval is highly successful in men with OA regardless of the cause of obstruction[62] and high live-birth rates (average of 34%) are achieved.[62] Neonatal data are also reassuring: Woldringh et al. in a follow-up study of 378 children born after ICSI with epididymal sperm have shown that ICSI with epididymal sperm does not lead to more stillbirths or congenital malformations in comparison to IVF and ICSI with ejaculated sperm.[63] A study by our group on neonatal outcome of 724 children born after ICSI using nonejaculated sperm showed no differences between children and fathers with NOA and OA.[64] The overall major malformation rate and incidence of karyotype anomalies were comparable between the nonejaculated and the ejaculated sperm groups (odds ratio [OR] 1.4; 95% confidence interval [CI], 0.9–2.2).[64] The risk of major anomalies in live borns does not depend on the origin of the sperm, which is in accordance with other groups.[65–67]

Men with NOA have dysfunctional testes resulting from a number of conditions: genetic and chromosomal abnormalities, postinfectious diseases, trauma, endocrine disorders, and idiopathic causes, and the vast majority of them have irreparable testicular failure. NOA patients undergoing TESE should be counseled that not only are the sperm recovery rates limited but also that the fertilization, implantation, and conception rates are decreased compared with men with normal spermatogenesis. Sperm retrieval rates after TESE range from 16.5% to 80%.[4] The differences in the retrieval rates arise from the inconsistencies in the selection of patients, e.g., inclusion of patients with hypospermatogenesis or patients without proper histopathologic diagnosis could lead to inclusion of patients with misdiagnosed obstructive azoospermia resulting in overestimated retrieval rates.[68]

There is scarce information about cumulative delivery rates using testicular sperm in NOA. The crude cumulative delivery rate after three ICSI cycles was 17% in the first report by our group,[69] which increased to 34% after three cycles and to only 37% after six cycles in the latest publication by our group.[68] Improvement in the ART laboratory as well as inclusion of frozen embryo replacements may account for the differences. Vloeberghs et al., in a 15-year follow-up, observed a clinical pregnancy rate per cycle of 21.7% and a live birth delivery rate per cycle of 20.6% in NOA patients. The important information provided by Vloeberghs et al. is that almost 4 out of 10 (37%) NOA couples who undergo ICSI treatment will have a delivery. However, unselected candidate NOA patients should be counseled, before undergoing TESE, that only one out of seven men (13.4%) will eventually father his genetically own child.[68]

Although the use of cryopreserved testicular sperm for ICSI has several advantages, the data concerning the outcomes of IVF-ICSI procedures using frozen–thawed testicular sperm are still controversial. A meta-analysis showed a significantly lower implantation rate when frozen–thawed sperm had been used compared with fresh sperm (relative risk [RR] 1.75; 95% CI: 1.10–2.80); however, no differences were observed in fertilization and ongoing pregnancy rates.[70] Our group has shown that despite the increased risk of not finding sperm suitable for injection, the outcome of ICSI cycles using frozen–thawed testicular sperm is not inferior to cycles with fresh testicular sperm.[68]

There is always a risk that following thawing there will be no sperm available for injection as was shown by Vloeberghs et al. In 27.5% of NOA ICSI cycles scheduled with frozen–thawed testicular sperm, back-up fresh TESE procedure was performed on the day of oocyte retrieval.[68]

Knowing that the surgical techniques of sperm retrieval have reached their efficiency plateau, what can be done to improve the sperm selection in the surgically retrieved samples?

At present, there are limited diagnostic tests available to evaluate the genetic integrity of a semen sample for both ejaculated and surgically retrieved sperm, as, traditionally, male fertility evaluation has used conventional semen parameters. DNA damage found in sperm can negatively affect treatment outcome.[71,72] There is an urgent need to identify novel markers that can assess the sperm genetic integrity. The effect of abnormal sperm chromatin on subsequent development will depend on the severity of the damage and the repair capacity of the oocyte.

There is a lack of selection against chromosomally abnormal sperm and increased sperm aneuploidy translates to increased aneuploidy in embryos.[73] In the case of chromosomal translocations, the high percentage of chromosomally unbalanced sperm is shown to translate to a high proportion of chromosomally

unbalanced embryos.[74] NOA patients have an increased risk of producing high levels of sperm aneuploidy. This is all thoroughly discussed in a recent review on the impact of sperm DNA chromatin in a clinic and current state of the ART.[75]

The perfect sperm selection test has yet to be developed. It has to be an easily applicable, reproducible method with high specificity, without any harmful additives, which can identify both viable and genetically normal spermatozoa. As demanding as this sounds there is a potential in Raman microspectroscopy that assesses membrane integrity and detects DNA damage of spermatozoa noninvasively and without destruction of the spermatozoon itself.[76]

Conclusion

The use of ICSI combined with sperm-harvesting techniques has moved frontiers in fertility treatment for azoospermic males. Sperm retrieval technique itself seems to have no impact on the success rates of ICSI. Patients with NOA have lower success rates in both sperm retrieval rates and live delivery rates compared with OA patients. One in seven men in an unselected NOA population has a chance to father a child.

The choice of both the sperm recovery method from surgical samples and sperm selection method depends on the embryologist and on the laboratory set-up. Each of the methods currently available is associated with both the advantages and disadvantages. The embryologist has the final say and will choose the most adequate method for the given setting.

After two decades, the struggle for improving treatment outcome in terms of success rates and safety in azoospermic men continues.

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