Reproductive Health and the Environment

Edited by
P. Nicolopoulou-Stamati, L. Hens
and C.V. Howard

Environmental Science and Technology Library

Springer

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Environmental Science and Technology Library

VOLUME 22

The titles published in this series are listed at the end of this volume.

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A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN-10 1-4020-4828-9 (HB)
ISBN-13 978-1-4020-4828-9 (HB)
ISBN-10 1-4020-4829-7 (e-book)
ISBN-13 978-1-4020-4829-6 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

www.springer.com

Printed on acid-free paper

Desktop publishing by Dao Kim Nguyen Thuy Binh and Vu Van Hieu.

Editorial Statement

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PREFACE

The scope of environmental impact on reproduction is very large. It aims at covering all aspects of reproductive problems and their links to the environment. Therefore, it deals with female and male reproductive impairments, whatever their severity and their sites, gonad or extra-gonad.

For women, effects include direct lesions of the oocyte, with possibilities of chromosomal abnormalities but also malformations of the reproductive tract. Reduced fertility and in some cases total infertility may also result from menstrual irregularities, which can at times include anovulatory cycles. Hormonal changes, even in the absence of menstrual irregularities may also lead to decreased fertility. In addition to congenital malformations, other impairments of the reproductive apparatus may occur, as in the polycystic ovarian syndrome or in endometriosis. Finally one can also include effects during pregnancy leading to spontaneous abortion, preterm delivery still birth or altered sex ratio. Impacts on age at menarche and age at menopause lead to a change in the duration of reproductive life.

For men, effects include poor semen quality, through low numbers or reduced motility or fertilizing ability of spermatozoa, and malformations of the reproductive tract. Reduced fertility may also result from hormonal changes at various levels of the hypothalamic-pituitary gonadal axis. As in women, malformations may be congenital or anomalies may be later developed, for example alterations of semen quality or hormonal imbalances. Specific conditions play a role such as cryptorchidism and more globally the testicular dysgenesis syndrome. Effects on the pregnancies of the partner can be included. Finally, the impact on age at puberty will affect duration of reproductive life.

The multiplicity of health effects leads to a challenge to correctly quantify the disease burden. Population-based registries of reproductive impairments do not exist as such. Registries of congenital malformations only cover a small proportion of potential endpoints. Therefore the correct descriptive epidemiology of reproductive problems in humans is still problematic. This has a direct impact on the evaluation of potential clusters in geographic epidemiology and on the estimation of time-trends in secular epidemiology.

Complementary and pertinent information comes from the study of reproductive life of animals, be they pets or farm animals or wildlife. Observational studies of animal reproductive functions may help in the elucidation of geographic differences or

time-trends. Experimental evidence coming from properly designed studies may confirm the role of suspected risk factors.

The definition of environment varies according to the person using the word. Usually, it refers to the physical, chemical and biotic factors that act upon an individual. The notion of the social and cultural conditions has then to be added. Some go even further and count behaviour among the environmental factors. Our choice is to focus on physical, chemical and when appropriate biological agents present in the surroundings of persons (or animals). Social and cultural factors may also be considered. By contrast, behavioural habits, such as smoking are generally excluded.

In order to establish links between reproductive problems and the environment, one needs to carefully assess exposures. A lot of the suspected agents are present in small quantities, for example as contaminants in food, water, soil or air. Even the establishment of the presence or absence of such a substance in the environment is uneasy. For a very long time, epidemiology relied solely on questionnaires to assess exposure. These are of limited value when enquiring into substances that are unknown to the population and estimates have to be derived from the use of proxies such as place of living, occupational category or data on use of products. Quantification of exposure is even more difficult. Two elements have to be taken into account: duration of exposure and daily dose. The use of proxies will better help for exposure duration than for daily values. Fortunately, the development of biological markers of pertinent exposures represents a big step forward. Laboratory determinations may be done at different levels. Exposures can be measured in the environment, such as for example the level of a given pesticide in the water at a specific location where a person is present; it will represent the external dose. This information is of value but not directly pertinent to the estimation of risk. The internal dose, i.e. the dose penetrating inside the body is more interesting and even more so is the biologically active dose, measuring the quantity of the product reaching the target cell and interacting with the elements of the cell, after having taken into account metabolic pathways, receptors issues, etc., The added challenge is that a one-point in time measurement is not sufficient to adequately characterise exposure. A lot of exposures to contaminants occur at low levels, yet these compounds accumulate in target tissues and the problem becomes one of measuring body burden to try to approach a correct estimate of the effect of cumulative exposure. When doing so, it might be particularly relevant to measure all products of interest, i.e. the parent compound but also its metabolites, some of which may be more biologically active than the original product. Also, in addition to individual products, it is worth to measure total exposure to a class of products, for example to

have an estimate of the oestrogenic burden of endogenous and exogenous origins. In fact, the question of interactions among products, be they synergy or antagonism, cannot be ignored.

A given dose will affect individuals in various ways. Clearly vulnerability will be dependent on many factors, genetic and acquired. It will also be affected by the interaction with other factors, such as diet, physical state, as well as concommittant exposures. It is now well known that there exist windows of opportunity, i.e. time periods during a person's life when the exposures will have a greater effect. These may vary according to the product but in general they correspond to uterine life and pre- and peri-pubertal period.

Despites all the above mentioned difficulties, a large body of evidence has become available as illustrated in the present book. Phtalates, pesticides, solvents, medical drugs have, among others, been linked to adverse reproductive outcomes. For a number of these products commonly called endocrine disrupters, the potential exists for not only reproductive effects but also other long-term outcomes, such as cancer. This poses the question of toxicity, including genotoxicity, carcinogenicity but also hormonal effects. This distinction is relevant for issues of dose-response and threshold.

The intention of *Reproductive Health and the Environment* to make all aware of the relation between environmental factors and reproductive impairment. Detailed descriptions of specific health problems and specific compounds are given in this book. State of the art knowledge will therefore be available to the readers. The following step is to encourage everyone to act. Faced with burden of evidence, be it convincing data from well-designed and conducted studies or even only reasonable suspicion of effect, one has to decide to protect public health. When needed, the precautionary principle will be applied and protection from potentially harmful exposures will be enforced. When deciding on the level of measures to be used, care will be taken to protect all, including the most susceptible, be they so for biological or social reasons. This needs to be urgently done as we now have increasing concerns due to increasing rates of the diseases. Against this background, and with a holistic view of the world, an urgent ecological approach is warranted. It is by acting now and protecting in particular the children that we will be able to see in ten to twenty years the preventive effects in terms of reduced morbidity and mortality for several diseases, not limited to the reproductive ones. The future of our population and our planet is in our hands.

A.J. SASCO

ACKNOWLEDGEMENTS

All papers of this book have been peer reviewed. The Editors are most indebted to the colleagues who reviewed various chapters of this book:

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The editors of this book wish also to thank the EU-SANCO and the Municipality Dikaiou Kos for their support to the A.R.E.H.N.A. project (www.arehna.di.uoa.gr), which provided the main scientific bases of this book.

The help in this book's language editing by the late professor Costas Evangelides, who passed away just before this book was published, is highly appreciated and his memory will be cherished.

The camera ready copy of this book was produced by Vu Van Hieu and Dao Kim Nguyen Thuy Binh. This work is most sincerely appreciated.

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INTRODUCTION: *ENVIRONMENTAL IMPACT ON REPRODUCTIVE HEALTH, RECENT TRENDS AND DEVELOPMENTS*

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Summary

The link between reproductive health and the environment has been strengthened by the findings of recent studies. It appears that environmental influences on fertility could be far greater than was previously thought. It becomes important to link results and data from these and other such studies into a model that facilitates explanations and predictions of phenomena, observed in the field. To this purpose, this book presents many of the environmental aspects that may affect female and male fertility. It focuses on the case of male infertility related to sexual organ maldevelopment such as hypospadias and cryptorchidism. Moreover it examines the relationship of semen quality to semen maturation while also addressing other aspects of fertility not related to semen.

Some of the studies in this book indicate correlations between exposure to defined levels of environmental pollutants and decreases in certain aspects of fertility. In this context, some known Endocrine Disrupters (EDs), such as PCBs, have been studied and compared with respect to their prevalence. In the female reproductive system, attention has been focused on oocytes and their maturation, this being a hormone dependent and therefore likely to be influenced by EDs. In addition other aspects of organ maturation, pregnancy and exposure to pesticides are discussed.

P. Nicolopoulou-Stamati et al., (eds.), Reproductive Health and the Environment, 1–19.

However, discussion is not restricted to pesticide pollution, important findings presented in this book, show that other substances such as BPA, can also affect reproductive health. The list of compounds includes some medical drugs, which have been capable of affecting children of both sexes. It is widely accepted that children are particularly vulnerable to such environmental exposures, affecting their reproductive capacity from an early stage. Wildlife studies can provide a useful framework for reference, as these systems can be studied in organisms that have been exposed by accident to significant amounts of relevant environmental pollutants throughout their natural life-spans.

The final sections of this book discuss the importance of communicating the results and messages that these findings generate to both the public and to decision makers. It indicates the need for policymakers to work closely with scientists, non-expert advisors, the media and the public in order to protect public health today and for generations to come. It is underlined that there is a strong need for applying the Precautionary Principle and adopting models as investigative tools that will allow us to assess the long-term as well as the short-term consequences of environmental pollution.

1. Introduction

Reproduction is a broad subject, with mechanisms and systems ranging from molecules to whole body systems. All living beings have a common biological fate, to reproduce or face extinction. In that respect, they are all dependent upon their environment, for the availability of the right conditions for individual survival are crucial.

The biology of reproduction is complex. To address it requires knowledge of multi-organ structure and function in diverse systems from brain – endocrine interactions to germ cell maturation, for example.

Organisms interact with their environment and this process affects the way they develop. When genes were first discovered it was thought that they simply did their job of expressing themselves in a slavish manner. What has been discovered increasingly in the past few decades is that the environment can directly influence gene expression in the embryo and during infancy, which can lead to changes in the final make up of the resulting individual or phenotype. That is to say, if normal development is disrupted or perturbated, and then there are consequences, most of which will be irreversible in the adult. As will become clear to the reader of this book, a number of conditions, including decreased semen quality and congenital

reproductive malformations, have been associated with exposure to EDs during the fetal period of life. It should be realised that, while pharmaceutical drugs usually need to be present in the adult in parts per million or parts per thousand to have a therapeutic effect, our hormones work in parts per trillion. In the embryo many processes of development are controlled by hormones at critical concentrations. Thus developmental disruption in the developing creature can occur at thousands or millions of times lower concentrations than those required to affect adults.

A variety of designs of studies have been employed to assess the degree of severity of environmental impacts on human reproductive health: case studies, epidemiological studies, biochemical, molecular and genetic studies, in addition there are studies conducted on wildlife. The findings of these studies pose many classes of questions. Some of these are specific: What are the environmental levels of particular pollutants which trigger adverse events and what should be the regulatory thresholds? Other questions are broader in nature: Which general policies should be developed to approach such complex problems? What is the best strategy to increase public awareness of such problems?

2. General considerations

Studies conducted in the field of environmental impact on reproduction have faced several difficulties. The problems centre on the question as to whether animals studied are physiologically sufficiently relevant models to be predictive for humans. Another consideration arises from whether the wildlife studies conducted are open to too many different confounding factors. This is to be compared and contrasted with investigations on laboratory animals, which usually have a very narrow scope, and therefore specific. Thus, they can be assumed not to be able to account for the entire range of phenomena observed in humans.

On the other hand, human studies are more limited both in their scope and availability than the other types of studies available, since they have to rely on population and epidemiological methodology, with all the strengths and weaknesses that are associated with those approaches. Another aspect that renders such studies difficult to conduct is the element of time that is usually required. A further problem that needs to be considered is the difficulty associated with identifying exposure, measuring it, and determining effects for the substances considered. The levels of some of these are low, but the systems where effects are exerted do not require great quantities (Brooks, 2000). However the challenge of this endeavour lies with

managing to draw valid results, and in choosing the methods by which to achieve this.

In order to identify a course of action and transform it into policy, it is necessary to recognize the extent of the problems that require a solution. For that, the causes and the consequences of phenomena need to be known, or at least assessed. Fertility, and environmental impacts on fertility, can be particularly difficult in that respect, as the complexity and interlinking of various factors defies most applied methods used to acquire knowledge (Claudio *et al.*, 1999). Also, the timescales involved, the required duration of informative studies, and the variety of possible systems involved are daunting to consider and apply when designing scientific investigations (Sharpe and Irvine, 2004). Some of the recent aspects are presented herein; for it is the aim of this book to review the recent trends and developments of environmental impacts on reproductive health, with the focus being on fertility. Furthermore, to expose the manners by which fertility can be assessed and affected, by presenting the most modern techniques and methods available in the field. Finally, it is hoped that the discussion and presentation of old and novel ideas will lead to the development of a more holistic and unified perception of the interaction between reproductive health and the environment.

3. Reproductive health and the environment

The importance of environmental impact on reproduction is not to be underestimated. Even before conception, from the production of gonads, to release of the oocyte, its fertilization, and the subsequent period of pregnancy and birth, reproductive health is subject to alterations and changes at any stage. Effects can be severe or subtle, isolated or multiple, at different periods (Barlow, 2001). The observed phenomenon can be the result of a complex network of environmental effects, and it is in this respect that it is necessary to maintain an open-minded approach to reproductive health problems that do arise (Feichtinger, 1991; Thonneau, 1993). It is vital not to rush to assumptions on the origin or causes of a disease or condition until all aspects have been considered (Daston *et al.*, 2003).

4. Epidemiological studies on semen quality and fertility

Semen quality has been noted in several studies to be decreasing across different populations (Swan *et al.*, 1997; Swan, *et al.*, 2003a). This has a corresponding impact on fertility. Sperm quality itself can be affected in many different ways: sperm numbers, motility of sperm, and capacity of fertilization of ovules,

necessitating a complex array of proteins. The lack of some of them can have a severe impact on sperm quality and fertility. The causes for decreasing sperm quality vary: they can be genetic, congenital, or even occurring during adulthood, when most of the systems required have matured and ought therefore to be impervious to major structural or functional changes. This last statement has been found to be inaccurate, as epidemiological studies indicate (Swan, 2003). It is therefore essential to perform epidemiological investigations on population-wide fluctuations, in order to identify trends and link them to possible causes. The direct identification of a specific chain of causation for a specific observation is difficult (Golden *et al.*, 1998). Laboratory animals can be useful in that respect, as many studies, especially those on mice, investigate the relationship between specific chemicals and their effects on sperm quality and its fertilizing ability (Xu *et al.*, 2004). However the link between those results and their extrapolation to humans can not be implemented, just by these studies, even though they offer more ground on which to base future theories. The main aim of such a pursuit would be to create a holistic model that would allow for the assessment of future risks, and would indicate guidelines for the management of current issues that have been identified and require intervention (Hayashi, 2005). In this book, Hauser and Meeker (2006) discuss the difficulties associated with studying the effects of various chemical agents present in the environment, such as PCBs, non-persistent chemicals and phthalates, on semen quality. The parameters studied can be multiple and diverse, but an association has been established between PCBs and semen quality through the use of epidemiological studies, particularly for sperm mobility. This study highlighted important mechanisms that link the environment with reproductive health, and also presents various other substances and chemicals that still require research. From a different perspective, Dhooge *et al*. (2006) investigated causes of male infertility, such as sperm quality, through different causes, such as occupational exposure. The epidemiological area chosen was Flanders, and the levels of environmental exposure were also assessed, with the presentation of biomarker assays of estrogenic activity, and how they operate.

5. Non-semen fertility aspects in man

Fertility is not solely linked to quality of sperm. The hypothalamic-pituitary gonadal axis is such an example, of control of the correct balance and maintenance of functioning of the sexual organs from the brain via hormones. Any disturbance of this axis can have repercussions onto the overall aspect of gonadal development, and hence fertility. Considering also the inter-relationship between these target organs and the brain, effects on these will affect the release of hormones, and ultimately the

brain itself. This aspect of fertility is extremely important in development, and will bear effects on the later reproductive ability of the adult, particularly in the male (Pflieger-Bruss *et al.*, 2004). Various chemicals, known as endocrine disrupters (ED), have been known to affect these crucial hormones, and affect both organs and/or brain development, leading to reproductive failure (Toppari *et al.*, 1996).

6. Potential impact of environment in male reproductive function: the example of cryptorchidism

Various congenital conditions, especially in the male, have been assigned to idiopathic causes: the case of cryptorchidism can be put forward. This was found not to be accurate, and studies have been conducted to identify potential factors that can cause these conditions (Skakkebaek, 2003). Many various explanations have been put forward, but some major ones can be traced back to the environmental impact that the parents, or the offspring, have been exposed to (Toppari *et al.*, 1996). The potential impact of the environment to male reproductive function has to be emphasized, in order to appreciate the sensitivity of the systems in question, and how they are affected. This in turn leads to a better understanding of epidemiological studies and of the mechanisms of causation of congenital diseases, such as cryptoorchidism, and their impact on fertility (Leissner *et al.*, 1999). As Huyghe and Thonneau (2006) discuss presently, there are signs and correlations that cryptorchidism is more common than previously thought, and that there seem to be risk factors involved in developing this condition. Some of these factors appear to consist of environmental EDs, which could cause the damage, or changes, resulting in the disease.

7. Experimental aspects of infertility

Fertility and infertility do not necessarily need to be considered as opposites, in the sense of deficiency: traditional view held that if some elements are lacking from the 'basal' condition of fertility, then the resulting state is infertility. The picture available now is far more complicated than this view would suggest. There are elements whose prevalence is reduced, and others that are enhanced. Indeed, infertility can be caused by over expression of production, or overcompensation, of some hormones or chemicals, and not necessarily the main or evident ones (Joffe, 2003). Effects can be subtle and can be linked to various secondary or peripheral systems that nevertheless impact on the primary systems of fertility (Murray *et al.*, 2001). Infertility can be considered as much of an independent state as fertility, not just the reduced aspect of fertility. It is in this sense that novel perspectives need to

be appreciated, and new experimental aspects have to be investigated in order to create models that can provide new explanations for the various conditions observed (Foster, 2003). It is vitally important to keep an open-minded approach to the framing of questions posed in studies, in order not to restrict the breadth and depth of knowledge that can be acquired.

8. Environment and women's reproductive health / Oocytes and ovarian follicles as targets of endocrine disrupters: consequences for reproductive health

Women's reproductive health is a crucial element of fertility. The first element would be the maturation of the primary sexual characteristics, the sexual organs, but also the correct growth and function of the gonads containing, the oocytes. Then, the secondary characteristics, such as breast development and ability to lactate, are essential. These can be targets of EDs: maturation and release of oocytes, from the stage of ovarian follicles, require the release of LH and FSH. Yet this phenomenon has been observed, independently from these hormones, under the effect of various endocrine disrupting chemicals (Pocar *et al.*, 2003). Any chemical or substance with the potential to affect these hormones would have an effect on the oocytes themselves, or even before them, the follicles. This would affect reproductive health, since if the oocytes are not sufficiently mature, they might not be viable enough to arrive at the uterus, or to be fertilized with sperm. And cells that are sensitive to estrogens have been found to be affected by various chemicals (Soto *et al.*, 1994).

The environment can also affect the various conditions that are necessary for correct implantation of the fertilized oocyte: this includes the fallopian tubes, the cilia present on the uterine tube surfaces, the walls of the uterus, the state of the mucosa and other membranes, and the ability of the mother to provide nutrients to the growing embryo. Problems could also occur from the shape of the organs, as observed in mice, affected by environmental chemicals (Timms *et al.*, 2005). The functioning of all these different elements require a tight coordination of hormone release, such as hypothalamic hormones such a GHrH, anterior pituitary ones such as LH and FSH, or sex steroids such as estrogen and progesterone. There are many other hormones involved which have important effects: an example would be oxytocin. It is plausible to assume that any interference would halt the process, or cause damage (Latini *et al.,* 2003; Sugiura *et al.,* 2005). Studies performed indicated that the fetus has a remarkable vulnerability to various chemicals that can be passed on from the mother's blood supply, as seen in rats (Bern *et al.,* 1992). The mother, therefore, can be subjected to influences from the environment in multiple stages. The reproductive stage is therefore far more sensitive than previously suspected and

the placenta barrier is not considered adequate for the protection of the embryo (Unadkat *et al.,* 2004). The oocytes themselves are discussed by Gandolfi (2006), indicating that even low levels of EDs can affect their maturation and cycle. Also, the level of knowledge of ED actions is discussed, while more studies are advocated into general mechanisms.

9. End products linked with polycystic ovarian syndrome

PCOS is a condition affecting the reproductive health and fertility of women, leading to various endocrinic disturbances. Its effects can vary: some of them are the development of androgenic features, resistance to insulin and anovulation. It is linked to genetics while being a very common endocrinopathy, and the exact mechanisms are still not understood (Diamanti-Kandarakis and Piperi, 2005; Diamanti-Kandarakis *et al.,* 2005). Due to the various endocrine-disrupting aspects specific to it though, it is a particularly interesting condition to relate to ED and environmental influences. The particular aspects of this condition, discussed in this book by Diamanti-Kandarakis *et al.* (2006), relates to the observation that these women present certain symptoms of infertility due to the accumulation of AGE products, whose origin is hypothesized to be environmental. The exact mechanisms are discussed, with emphasis on collagen formation and the consequences for endothelial dysfunction, potentially playing a major role in the causation mechanism of infertility observed in this syndrome.

10. Environmental exposure/ Foetal exposure on endocrine disrupting agents: the relation between hypo fertility and cancer

We live in an environment that has been greatly modified in the last hundred years. Humans distinguish themselves for having bypassed the straight interdependence from the environment, freeing themselves from its constraints on their growth and proliferation. Yet the rapid development of technology at the beginning of the twentieth century led to the application and dispersal of thousands of tons of novel, synthetic chemicals and compounds that were used in different sectors, from agricultural chemicals to industrial plastics. These contaminants have managed to spread worldwide and are not restricted just to their area of production, as some recent studies have shown from the exposure of PCBs in the deep sea (De Boer *et al.,* 1998). Even though the possible consequences of some of these substances have been recognized, and their production halted, as with DDT in most countries, their presence is still being detected, with surprising distribution patterns. More alarming, the parent compounds might have broken down to metabolites, whose effects are

more potent, or even combine their effects synergistically with other substances (Rajapakse *et al.,* 2002). This leads to hitherto unsuspected possible combinations, whose range and extent of action is almost impossible to estimate: therefore, these chemicals, broadly known as EDs, are not to be underestimated as a mechanism for causation of reduced fertility (Kaiser, 2005).

Moreover, as stated before, the fetus is particularly sensitive to some of these EDs, and the interrelationship between sexual organs, the brain and hormones mediating communication between them has been emphasized several times. Another phenomenon known to occur is that some of these substances, such as DDT, are lipophilic and bioaccumulative: they are absorbed and concentrated into fatty tissues of organisms. In the case of adults, this does not lead to any significant risk at low doses. Yet when it concerns mothers, their adipose reserves are mobilised for the energy consuming process of pregnancy: these EDs, accumulated in the mother, are then passed on to the fetus (Cohn *et al.,* 2003). The lack of any metabolic or excretion mechanism, due to lack of maturation, means that the 'inherited' chemicals will be present in an organism which is not prepared to deal with them and can cause permanent harm (McLachlan, 2001). This can take two aspects: the future reproductive ability of the fetus is impaired by damaging or inhibiting the genital organs or their development, or cells that are extremely prone to mutagenesis at this stage of development can become cancerous. This would correspond to the 'critical windows' of development, periods when the developing organism is highly malleable and dynamic (Silbergeld and Patrick, 2005). For instance, estrogen-sensitive cells could be activated by increased amounts of xenoestrogens, EDs that mimic these substances, or increase their production (Schettler *et al.,* 1999). Estrogenic effects are known to require relatively small doses that can cause major effects, especially in developing organisms; whereas studies in toxicology using adult animals and much higher doses would not detect them (Welshons *et al.,* 2003). The possible mechanisms need to be investigated, even though their inherent complexity is daunting: only then will we be able to acquire an appreciation of the mechanisms and their range involved in these processes.

11. Reproductive toxicology of pesticides: identification of hazards for female reproduction

Pesticides present a special case of environmental impact. Chemicals that have been manufactured for agricultural use, they were produced massively during the beginning of the twentieth century. Apart from the fact that they are suspected to be quite dangerous, the modus operandi of some of those chemicals are similar to that

of EDs. Many of them have been classified as such and their literature is quite extensive (Tilson, 1998). They are most interesting because they provide a manner on which to base more specific epidemiological studies, as the area where they are used, or produced, can be monitored for subsequent phenomena indicating altered fertility patterns in a prognostic manner (Cavieres *et al.,* 2002). Alternatively, areas where certain patterns of fertility impairments or congenital disorders start appearing on a population scale, the profile for recent pesticide use can be drawn, and examined. If a condition or phenomenon seems to correspond with increased or high use of a certain pesticide, or even a combination thereof, the correlation can be made with other areas where the usage of these products is prevalent. Such a pattern and scheme has been proposed, concerning semen quality (Schwan*,* 2003b). If the patterns of exposures and effects seem similar, animal models can then test the suspected agents to identify their modes of action and their range (Hayes *et al.,* 2003). Population studies render the specific identification of an origin for a condition particularly difficult; therefore any step that can be taken to elucidate a possible link in a chain of causation is valuable: the use of animals in combination with human studies is particularly useful (Younglai *et al.,* 2005). Foetal deaths have been observed during exposure to pesticides; with the argument that more detailed studies are necessary, the implication in these findings still do not allow disregarding the possible consequences (Arbuckle and Sever, 1998).

Such a comprehension of the possible effects of pesticides can be particularly useful when attempting to determine the likely events occurring in female reproductive health exposed to a constant source of them. This in turn allows a better understanding of how EDs can function. Studies on populations have been performed on women who have been exposed to pesticides, either through diet (Buck *et al.,* 2000) or by occupational exposure, of them or their spouse's (Greenlee *et al.,* 2003). The amounts of pesticides detected in these women, especially in more rural areas, where these substances have been used, can be surprisingly high (Schreinemachers, 2003). A study by Mantovani (2006) discusses and presents results of assessments of the different effects of pesticides presenting EDs function. The difficulties for establishing reliable methods for assessing these effects are also presented. Identifying the hazards of these substances to the reproductive health of women is imperative, as their toxicology can present severe symptoms and effects, even at very low doses.

12. Non-pesticide endocrine disrupters and reproductive health (PCBs)/ Impact of bisphenol A on fertility

The possible exposure from environmental sources of EDs can therefore arise from pesticides. As noted before, ED action can also occur from substances that become present in the environment, either in their original structure, or their metabolites. They can prove to be more elusive than pesticides, in the sense that correlation of epidemiological results and their presence is not so clear to determine in a geographic area. These compounds can migrate along long distances before causing effects in a particular area. By breaking down and mixing with other similar substances, their specific origin is almost impossible to trace. Their effects are far from being straightforward to assess (Hauser *et al.,* 2004b). Corsolini (2006) presents in this book the reasons outlined above: also, all EDs that are not pesticides can be ascribed to this category, and correspondingly affect reproductive health. Synergy, and the need to regulate emissions of these substances before release into the environment, is advocated. Bisphenol A (BPA) has been shown to possess an impact on fertility as well, through its actions on reproductive features such as development of mammary glands in mice (Munoz-de-Toro *et al.,* 2005). Studies on its effects and mechanisms are continuing and will probably in the near future provide us with pieces of the puzzle.

13. Medical drugs impairing fertility

Fertility has also been shown to be affected through medical prescriptions and drugs. The effects once again can take the pathways ascribed to most EDs, especially when side effects have not been evaluated. Usually problems arise due to the long-term effects these drugs can have, which are simply not envisaged when short-term toxicological tests are conducted for safety of use. The case can be illustrated by diethylstilbestrol (DES), a synthetic drug, whose effects were only detected twenty years after its introduction (Fowler and Edelman, 1978), in the offspring of pregnant women, necessitating new methods to detect and treat them (Vieiralves-Wiltgen and Engle, 1988). These cases are not isolated but well documented studies can unfortunately only be conducted through large populations of exposed individuals, which do not occur often. Other iatrogenic impacts on fertility specifically attributable to drugs can be those that affect pregnant women, particularly during those periods when the fetus' development is most vulnerable. Also, in males and females, some of these drugs can affect gonad quality and reduce subsequent fertility and effectiveness of reproduction. Phthalates have been used in certain medications (Hauser *et al.,* 2004a). Although not one of the most common paths by which fertility can be affected, it remains though one for which the source is

relatively easily identifiable, provided correct records of patients are kept. Pandiyan (2006) discusses in this book the possible underlying mechanisms, while also underlining the difficulty of assessing cases. Most couples that are taking medications might not even be aware of decreased fertility being linked to iatrogenic causes, and just ascribe it to themselves, or sheer bad luck. The difficulty, as presented by the author, is in maintaining a judicious dispensing of drugs, and also not immediately jumping to conclusions from manifestation of diseases, as the cause cause of infertility can be due to a mixture of both. In this sense, information provided by these drugs can help our understanding of the effects of EDs that present similar effects, and also how to prevent or treat their effects, as some of them do not cause irremediable damage.

14. Reproductive effects from exposure to environmental mutagens. The impact of chemicals on children: effects to reproduction systems

Environmental mutagenic effects constitute a special feature of certain EDs. Instead of possessing acute or subtle chronic effects on a hormonal system entraining physiological changes, substances can also affect genes and gene expression. This can have as a consequence the deletion of genes, mutations, and other alterations to the genotype. The result can be silent, due to the high degree of redundancy of the genome, or it can have an effect. On the other hand, the result can be cancer. This kind of effect is particularly likely in the genital organs and their cells, the germ line cells, due to their high natural proliferation rate (Favor, 2005). The various aspects of mutagenesis have to be considered in more detail, as their effects can be wide-ranging and not necessary attributable to EDs and their actions, even if they are caused by them (Marchetti and Wyrobek, 2005).

Children are once again particularly susceptible to chemicals, both because of the ubiquitous distribution of these chemicals, but also due to their vulnerability to them (Brent and Weitzman, 2004). An example is provided by polybrominated biphenyl exposure *in utero* and postnatally for girls, causing a premature menarche, with all the problems that are inherent in physiological phenomena occurring before the normal time for maturation of the relevant system (Blanck *et al.,* 2000). A chapter by Rubes (2006) presents two studies on air pollution, and how the carcinogens present affect the reproductive health and fertility of two different groups, young men and pregnant women in the Czech Republic.

15. Wildlife studies on fertility and exposure to pollutants

Studies on fertility are complicated by the fact that it is very difficult to estimate single causes for particular phenomena observed. Laboratory studies can analyze particular elements, but are far from being representative of the real situation in the environment. They are also not very efficient in investigating patterns of exposure, unless there is a vast amount of resources and time invested in them (Brent, 2004). Wildlife studies allow bridging the gap between human epidemiology and laboratory findings and are useful in creating working models that can then be tested in the laboratory. Also, most of the effects and exposures are either underway, or have already been done. For this reason, they provide an attractive alternative to gain better understanding of how the environment can affect species. The wide variety of species that can be studied is another advantage. Also, some of the exposures seen in Wildlife would be considered unethical for application in human subjects, but also laboratory animals. An example would consist in the effects seen in the contamination of the lake Apopka, and the resulting effects on alligator reproductive functions (Guillette *et al.,* 1994). Therefore, the case studies offered provide insights on long-term exposures that would be too costly and questionable to carry out in the laboratory *de novo*, while still allowing drawing useful mechanisms and hypotheses for future research.

16. Problems in assessing low dose effects of endocrine disrupters - Policy and policy instruments, the precaution on the regulation of chemicals

The essential problem of EDs is to determine their range of effects. This can be difficult since it can vary according to the dose which has effects at the picomolar concentration (Wozniak *et al.,* 2005). Also their effects cannot be determined by using classical approaches, such as in toxicology, since they affect hormones. Hormonal pathways have a different mode of action than toxic chemicals: their effects can be stronger at lower doses than at higher doses. These facts attest to the difficulty of the task and advocate for the use of enhanced sensitivity assays to detect their presence and estimate their range of effects. There are various considerations that need to be taken into account in this respect, both theoretical and practical (Tingen *et al.,* 2003).

The findings from these studies need to be assessed, and included into a body of knowledge, which could then be used in order to draw out relevant issues necessitating implementation of policy and management. It is in this respect that policy instrument is considered with the current thoughts and trends on this subject.

The best policy to be implemented is the application of the Precautionary Principle. Gies (2006) discusses in this book the relevant issues and problems with assessing ED effects, and how results are perceived by regulatory bodies. A direction which is much safer and simpler to apply consists of a careful screening system, with more funds to research more sensitive assays and screening tests, before introducing a new compound or substance, then by releasing it into the environment without possibly realizing the consequences of such an act. The short-term approach must not prevail over long term planning and consideration. If the Principle is itself applied in the designing of new drugs through the various cycles of tests and checks and trials, why should not the same be applied for substances that are liable to end up in the environment? Furthermore the dramatic case of the DES incident, raised awareness about the dangers of applying substances without framing the correct questions.

17. How can the 'fertility and environment' message be disseminated (getting the message across)

There are certain target groups of individuals that are concerned by issues such as environmental impact on reproductive health and fertility. These are the general public, scientists, non-expert professionals and policy makers. Policy makers are funded by the public. Non-expert professionals are in a prime position in this debate, in the sense that they straddle the line between the science, observing consequences and cases first hand, while also being at a position to create and support results, informing the public and policy makers. Scientists ought to inform policy makers and the public of their results and predictions, so that they can be equally disseminated, with the collaboration of non-expert professionals, who have a more direct link to the people that are under risk. The major difficulty in the last fifty years has been that policy makers on their own have a difficult time applying the changes that are sometimes required. This is partly due to misinformation on the issues involved, but sometimes can be attributed also to a short-term perspective of phenomena. In that respect, the public have been more efficient in getting the message across but in order to do that, they require the proper level of information. Communication between those groups is essential. Furthermore new and more efficient ways of educating and presenting the information obtained from research have to be elaborated and applied. Illig and Lyons (2006) present in this book the issues at stake, which affect society as a whole. For these reasons, the environmental message concerns everyone, and is a sensitive subject that has to be dealt with concern and accuracy, requiring special attention from all parties involved, but particularly the public and policy makers.

18. Conclusions

The impact of the Environment on Reproductive Health is a difficult topic. The changes that occur to the organs and systems involved in reproduction are multiple and subtle. They do not necessarily present a simple pattern of expression therefore their identification is extremely time and resource consuming. What is indubitably seen through the studies presented in this book is that reproductive health is not a static system that is liable to disruption only through certain well-defined mechanisms, while having a plethora of idiopathic conditions on the side. The environment can have multiple effects, many more than previously realized, which can cause serious consequences. A direct correlation is difficult to draw, but strong links have been shown in this book between environmental exposure to certain chemicals and infertility. Reproductive health state and intact fertility represent a brief overview of the necessities for efficient reproduction. It is staggering to realize how many of these steps can be disrupted at the molecular level, resulting in infertility and/or reproductive failure. Another important fact that requires to be remembered is that some of these effects can be completely masked or not easily detected at all. Misdiagnosis or ignoring the results that are available from epidemiological studies, is quite common. Congenital diseases caused by EDs can therefore be missed, and the incidence of exposure can be estimated as much lower than it actually is: this book presents those aspects, and discusses approaches that can be taken, and research needing to be done.

This book's main objectives are to disseminate information on the environmental impact on reproductive health to non-expert advisors, by assisting in the implementation of strategies and policies necessary to avoid any risks from exposures. Waiting for environmental disasters to happen, particularly involving fertility and reproduction, is a mistake that society could not afford to make, as the economic and ethical damages will be incalculable.

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SECTION 1:

THE LINK BETWEEN REPRODUCTIVE HEALTH AND ENVIRONMENT

EPIDEMIOLOGICAL STUDIES ON THE RELATIONSHIP BETWEEN SEMEN QUALITY AND ENVIRONMENTAL CHEMICALS: *HISTORIC AND CONTEMPORARY COMPOUNDS*

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Summary

Recent reports of downward trends in semen quality and increased rates of developmental urogenital tract anomalies and testicular cancer have raised both scientific and public concern about the potential risk of environmental chemicals to male reproductive health. Of particular concern is whether some contemporary use environmental chemicals alter semen quality. Semen quality refers to both conventional measures, such as sperm concentration, motility and morphology, as well as sperm DNA integrity as assessed by bioassays. Specific toxicants of interest include phthalates and pesticides (primarily insecticides and herbicides), as well as polychlorinated biphenyls (PCBs). The human data on the relationship of semen quality with phthalate and pesticide exposure are limited and does not currently allow for a definitive conclusion on whether adult exposure, at background environmental levels, alters semen quality. However, the epidemiologic data support an inverse association of PCBs with reduced semen quality, specifically reduced sperm motility. The associations found were generally consistent across studies despite a range of PCB levels. In addition to the chemicals discussed in detail, there are additional classes of chemicals that require further study as to their relation with

P. Nicolopoulou-Stamati et al., (eds.), Reproductive Health and the Environment, 23–56.

human semen quality. These chemicals include alkylphenols, such as 4-nonylphenol, bisphenol A and the fluorinated organic compounds.

1. Introduction

Scientific and public concern about the potential risk of environmental chemicals to male reproductive health has been driven by reports of temporal downward trends in semen quality (Carlsen *et al.,* 1992; Swan *et al.,* 2000), increased rates of development anomalies, specifically hypospadias and cryptorchidism (Paulozzi, 1999), and increased rates of testicular cancer (Adami *et al.,* 1994; Bergstrom *et al.,* 1996; Huyghe *et al.,* 2003). Furthermore, a recent study among healthy young men found an unexpectedly high proportion of poor semen quality (Andersen *et al.,* 2000). These observations raise the possibility that human exposure to environmental chemicals may partially be responsible.

The assessment of semen quantity and quality is used clinically to assess potential fertility (WHO, 1999) and in epidemiological studies as a biomarker for the potential effects of toxicants on the male reproductive system. Semen quantity and quality may be altered through toxicant affects on the neuroendocrine system (i.e., the hypothalamic-pituitary-testis axis), the testis (which includes Sertoli and Leydig cells as well as the spermatogenic cells), and on post-testicular sites such as the epididymis. Potential toxicants may affect semen quantity and quality by interacting with or disturbing one or more of these targets.

Although semen quality is measured in the adult male, it may be affected by exposures during various life stages, such as during gestation, puberty, or as an adult. In addition, as recently shown by an elegant study by Anway *et al.* (2005), there are transgenerational effects of chemicals, whereby exposure of the maternal or paternal (or even the grandparents) gametes to chemicals may confer an increased risk of altered semen quality in the offspring. Although early life exposure may impair spermatogenesis, as supported by evidence from studies in laboratory animals and human studies on prenatal exposure of men to DES, the human data is generally limited to the assessment of both semen quality and environmental or occupational exposure during adult life. Therefore, the present chapter largely describes evidence on the relationship between adult exposure to environmental chemicals and semen quality. It is anticipated that evidence on exposure during early life stages, such as gestation and puberty, will become available in the coming years.

In the present chapter, we focus on epidemiologic studies that explored the relationship of semen quality with several contemporary use environmental

toxicants. Specific toxicants include the following: phthalates, pesticides (primarily the contemporary use non-persistent pesticides), bromopropane and polychlorinated biphenyls (PCBs). Although PCBs are not currently in use, we included PCBs in the chapter because there are many recent publications worthy of discussion.

The majority of epidemiologic studies on the environment and semen quality are cross-sectional designs in which exposure and semen parameters were assessed at a single point in time. This makes it difficult to assess causation because it is not always clear that exposure precedes outcome. In addition, the reversibility or permanence of the effect, if one is present, is also difficult, if not impossible, to determine. It is well-known that semen quality parameters, such as sperm concentration, motility and morphology, vary both between as well as within individuals. The variability in semen parameters may be related to biological and/or social factors (such as abstinence time). Because of the within individual variation in semen parameters, the collection of a single semen sample makes it difficult to assess the relationship between chemical exposures and human semen parameters. The intra-individual variability will generally tend to bias associations, if present, to the null hypothesis. Another limitation of most human semen quality studies is the low participation rates, often well below 50 per cent. This may introduce selection bias if men agree to participate based on knowledge of both exposure and outcome (semen quality). In addition, some epidemiologic studies may not collect information on important potential confounders and/or may not have adjusted for confounders.

Because this chapter is not an exhaustive review of the epidemiologic literature on environmental and occupational toxicants and semen quality, the reader is directed towards previously published reviews and book chapters. These earlier publications discuss known human male reproductive toxicants, primarily occupational agents, such as 1,2-dibromo-3-chloropropane (DBCP), diethylstilbestrol (DES), inorganic lead, alkylating neoplastic agents, ethylene glycol, monomethyl and monoethyl ethers, carbon disulphide, ethylene dibromide, and ionizing radiation (Rosenberg *et al.,* 1987; Bonde and Giwercman, 1995; Lahdetie, 1995; Tas *et al.,* 1996; Figa-Talamanca *et al.,* 2001; Pflieger-Bruss *et al.,* 2004; Vidaeff and Sever, 2005).

2. Non-persistent pesticides

The term "non-persistent pesticides" (also commonly called “contemporary-use pesticides”) refers to chemical mixtures that are currently available to control insects (insecticides), weeds (herbicides), fungi (fungicides) or other pests (e.g.

rodenticides), as opposed to pesticides that have been banned from use in most countries (e.g. many of the formerly popular organochlorine pesticides such as DDT). Some common classes of non-persistent pesticides in use today include organophosphates, carbamates, and pyrethroids. Though environmentally non-persistent, due to the extensive use of pest control in these various settings a majority of the general population is exposed to some of the more widely used pesticides at low levels.

There are several epidemiologic studies on men exposed to non-persistent pesticides during agricultural work. A cross-sectional study on testicular function measured sperm concentration, motility, and morphology in 122 greenhouse workers defined as low, medium or highly exposed to more than a dozen pesticides (Abell *et al.,* 2000). Adjusting for abstinence time and other potential confounders, a higher proportion of abnormal sperm were found in the high exposure group compared to the group with low exposure. Lower median sperm concentration was also observed in workers with more than 10 years of work in the greenhouse compared to men with less than 5 years of experience. In a cross-sectional study on traditional and organic farmers, Juhler *et al.* (1999) investigated the relationship between dietary exposure to pesticides and semen quality. Estimating exposure through food frequency questionnaires and data from pesticide monitoring programs, the authors found that men with a lower intake of organic food had a lower proportion of normal shaped sperm according to the strict criteria (2.5% versus 3.7%, p-value = 0.003). However, organic food intake was not associated with the other 14 semen parameters measured in the study. Results in the study were adjusted for age, urogenital tract disease, spillage, abstinence time, smoking, and alcohol intake. Oliva *et al.* (2001) investigated the impact of environmental factors on infertility among 177 men in Argentina. Adjusting for age, BMI, abstinence time, income, health center, and smoking, a dose-related response was observed in (primary) infertile men occupationally exposed to pesticides. Significantly elevated odds ratios (95 per cent confidence intervals) were reported for sperm concentration (less than $1x10^6$/mL; OR = 3.4 (1.2, 7.4)), motility (<50% motile; OR = 3.6 (1.1, 11.4)), and morphology (<30% normal; OR=4.1 (1.4, 12.0)) for men exposed to pesticides compared to occupationally non-exposed men. Conversely, in models adjusting for many of the same variables, Larsen *et al.* (1998) found only marginal differences among 15 semen quality parameters from Danish farmers who sprayed pesticides compared to farmers that did not spray pesticides. These studies show a possible association between pesticide exposure and human semen quality. However, the non-specific assessment of pesticide exposure makes it difficult to determine which pesticides, if any, were responsible for the observed effects.

Few studies have been conducted that provide information on specific chemicals or classes of non-persistent pesticides and altered testicular function. Padungtod *et al.* (2000) studied the relationship between occupational exposure to organophosphates (parathion and methamidophos) and testicular function among Chinese pesticide factory workers. They found a significant reduction in adjusted mean sperm concentration (28.5 vs. 49.4 million sperm/mL; p-value = 0.01), and percentage of motile sperm (64% vs. 74%; p-value = 0.03) in the 20 exposed workers as compared to the 23 unexposed workers. In a recent Japanese study, pesticide sprayers exposed primarily to organophosphates and pyrethroids showed spraying season-dependant reductions in motile sperm velocity measures compared to unexposed controls (Kamijima *et al.,* 2004).

Two publications reported the results from a study on a small cohort of men exposed to carbaryl (1-naphthyl methyl carbamate; commonly known as Sevin®) during the production and packaging of the insecticide (Whorton *et al.,* 1979; Wyrobek *et al.,* 1981). Although analyses using sperm counts as a continuous measure failed to find significant differences based on carbaryl exposure, the authors found a greater proportion of oligozoospermic men among the carbaryl workers as compared to the chemical workers (Whorton *et al.,* 1979). In a subsequent publication on the same cohort of carbaryl production workers, Wyrobek *et al.* (1981) studied the relationship between sperm shape abnormalities and carbaryl. Morphological analyses showed an elevated percent of abnormal sperm in carbaryl workers as compared to comparison subjects, which remained after stratifying on potential confounders such as smoking, medical history, or previous exposure to hazardous agents. The proportion of men defined as teratospermics (greater than 60% abnormal sperm) was higher among the carbaryl workers than in the comparison group (28.6% and 11.8%, respectively).

More recently, researchers have utilized urinary and serum biomarkers of pesticide exposure to explore associations with reduced semen quality. In a US study on the male partners of pregnant women, Swan *et al.* (2003) compared urinary levels of pesticide biomarkers in 34 men with sperm concentration, motility and morphology below the median (defined as cases) to 52 men with above median semen parameters (defined as controls). They found elevated odds ratios [OR (95% confidence interval)] for alachlor mercapturate [30.0 (4.3, 210)], 2-isopropoxy-4-methyl-pyrimidinol (IMPY; diazinon metabolite) [16.7 (2.8, 98)], atrazine mercapturate [11.3 (1.3, 99)], 1-naphthol (carbaryl and naphthalene metabolite) [2.7 (0.2, 34)] and 3,5,6-trichloro-2-pyridinol (TCPY; chlorpyrifos metabolite) [6.4 (0.5, 86)]. However, a small study size led to the wide confidence intervals that restrict interpretation of the study results.

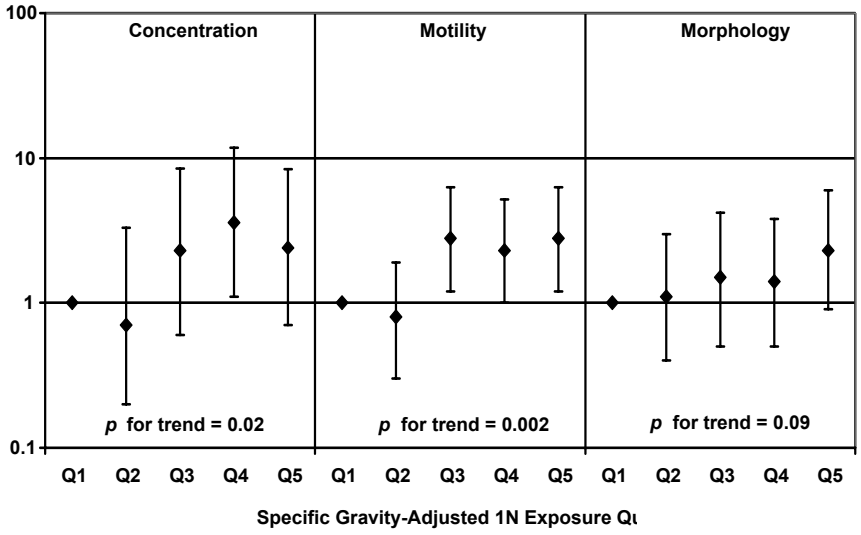

Figure 1. Odds ratios (95% confidence intervals) for the relationship between semen parameters and specific gravity-adjusted 1-naphthol (1N) exposure quintiles (from Meeker *et al.,* 2004a)

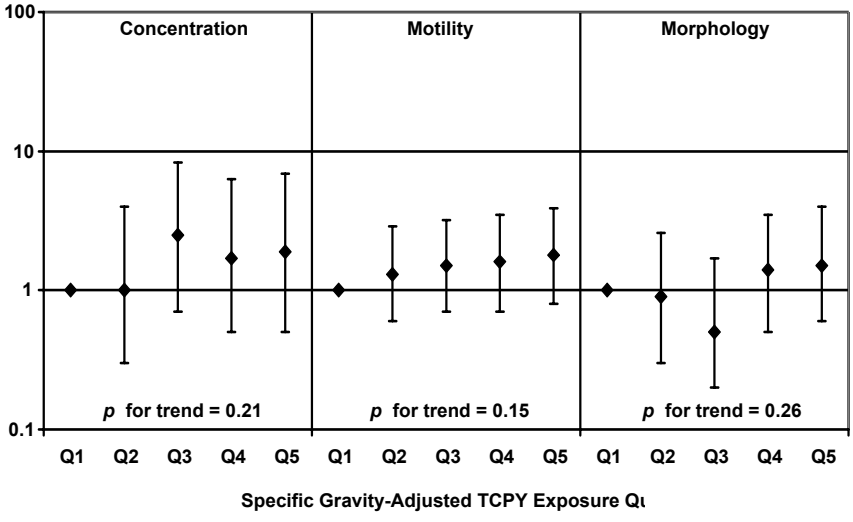

Figure 2. Odds ratios (95% confidence intervals) for the relationship between semen parameters and specific gravity-adjusted TCPY exposure quintiles (from Meeker *et al.,* 2004a)

Using urinary biomarker data representative of low environmental levels of pesticides commonly encountered among the general population, Meeker *et al.* (2004a) studied 272 men that were partners of an infertile couple. They found inverse associations between urinary levels of 1-naphthol, a metabolite of both carbaryl and naphthalene, with sperm concentration and motility (Figure 1). They also found a suggestive inverse relationship between the urinary metabolite of chlorpyrifos and sperm motility (Figure 2). When insecticide metabolite levels were categorized into tertiles, odds ratios (95% confidence interval) for medium and high tertiles of 1-napthol were 4.2 (1.4, 13.0) and 4.2 (1.4, 12.6) for below reference concentration (<20 million sperm/mL), and 2.5 (1.3, 4.7) and 2.4 (1.2, 4.5) for below reference motility (<50% motile sperm). In multiple linear regression analyses, an interquartile range increase in 1-naphthol (1.8 to 5.0 μg/L) was associated with a 3.9 per cent (-7.3, -0.5%) decline in proportion of motile sperm and a 16 per cent (-29, +1.0%) decline in sperm concentration. An interquartile range increase in chlorpyrifos metabolite (TCPY; also 1.8 to 5.0 μg/L) was associated with a 2.2 per cent (-5.1, +0.7%) decline in motile sperm.

Several studies have also explored the relationship between pesticide exposure and novel markers of male reproductive endpoints that extend beyond the conventional semen parameters. Recent human studies have reported increased sperm DNA damage associated with environmental exposure to carbaryl and the organophosphate chlorpyrifos (Meeker *et al.,* 2004b), and sperm genotoxicity following occupational exposure to carbaryl and the synthetic pyrethroid fenvalerate (Xia *et al.,* 2005; Bian *et al.,* 2004; Xia *et al.,* 2004). Additional studies have reported associations between organophosphate exposure and increased frequency of human sperm aneuploidy (Padungtod *et al.,* 1999; Recio *et al.,* 2001) and altered sperm chromatin structure (Sanchez-Pena *et al.,* 2004).

In summary, there are limited human studies supporting an association between altered semen quality and non-persistent pesticide exposure, specifically some herbicides and insecticides. However, the majority of studies were occupational studies involving simultaneous exposure to several pesticides. Thus, there is limited evidence on the relationship between male reproductive health endpoints and specific non-persistent pesticides. Furthermore, our understanding of low-level environmental exposure to non-persistent pesticides, primarily through diet and residential use, is not well understood. Additional research using biomarkers of exposure to specific pesticides is needed to further our understanding of the potential reproductive health risks associated with non-persistent pesticides.

Table 1. Summary table of epidemiologic studies (in chronological order) on the relationship between non-persistent pesticides and semen quality

Author, country	Study population	Exposure	Results	Comments
Whorton *et al.,*1979 US	47 carbaryl production workers plus 90 unexposed controls	Subjective exposure classification based on job tasks	Greater proportion of oligozoospermic men among the carbaryl workers (15%) as compared to the chemical workers (5.5%; p-value=0.07).	No adjustment for potential confounders. Sperm motility not measured.
Wyrobek *et al.,*1981 US	50 carbaryl production workers plus 34 unexposed controls	Exposure ranks/groups based on job type held for previous year	Elevated percent of abnormal sperm in carbaryl workers (52%) as compared to comparison subjects (42%; p-value<0.005). The proportion of men defined as teratospermics (greater than 60% abnormal sperm) was higher among the carbaryl workers (28.6%) than in the comparison group (11.8%; p-value=0.06).	Confounders considered: smoking, medical history, previous exposure to hazardous agents
Padungtod *et al.,*2000 China	43 Pesticide factory workers; 20 high exposed and 23 with no or very low exposure	Occupational exposure to ethyl parathion and methami-dophos	Exposure associated with reduction in sperm concentration and motility, but not sperm morphology. Adjusted means for exposed and non-exposed workers were: 28.5 and 49.4 million sperm/mL (p-value=0.01), respectively, for sperm concentration; and 64% and 74% (p-value=0.03), respectively, for percentage of motile sperm.	Confounders considered: age, abstinence period, current smoking status.

Table 1. continued.

Author, country	Study population	Exposure	Results	Comments
Swan *et al.* 2003 US	86 male partners from couples attending prenatal clinic	Urinary levels of pesticides or metabolites (IMPY, 1N, TCPY, others)	Increased odds ratios (95% CI) for below reference semen parameters associated with high exposure group for alachlor mercapturate 30.0 (4.3, 210); IMPY 16.7 (2.8, 98); atrazine mercapturate 11.3 (1.3, 99); 1-naphthol 2.7 (0.2, 34); and TCPY 6.4 (0.5, 86).	Small study size limited statistical power; odds ratios were unadjusted for potential confounders.
Meeker *et al.,* 2004a US	272 male partners from couples attending infertility clinic	Urinary levels of insecticide metabolites (1N, TCPY)	Inverse association between urinary carbaryl metabolite (1N) and sperm concentration, motility. IQR increase in 1N associated with 16% decline in sperm concentration and 3.8% decline in motile sperm. Suggestive inverse association between chlorpyrifos metabolite (TCPY) and sperm motility.	Confounders considered: age, BMI, abstinence time, smoking status, race, season
Meeker *et al.,* 2004b US	214 men from couples attending infertility clinic	Urinary levels of insecticide metabolites (1N, TCPY)	Using the neutral comet assay to assess DNA damage in human sperm, found associations between urinary carbaryl and chlorpyrifos metabolites (1N, TCPY) with percentage of DNA in the comet tail (p-value=0.0003 and 0.004, respectively).	Confounders considered: age, BMI, abstinence time, smoking status, race, season

Table 1. continued

Author, country	Study population	Exposure	Results	Comments
Xia *et al.,* 2005	16 carbaryl-exposed workers and 30 controls	Men defined as exposed or unexposed based on job tasks and air monitoring	Men with high occupational exposure to carbaryl (>5 mg/m^3 according to air monitoring) had a higher percentage of sperm with fragmented DNA (21±9) compared to internal (13±12; p-value=0.04) and external (14±7; p-value=0.03) controls.	Several potential confounders considered for subject eligibility into study (health, age, smoking, alcohol), but not included in the models

Abbreviations: 2-isopropoxy-4-methyl-pyrimidinol [IMPY]; 1-naphthol [1N]; 3,5,6-trichloro-2-pyridinol [TCPY]; confidence interval [CI]; body mass index [BMI]

3. Solvents

Organic solvents are widely used for cleaning in industrial production processes and are also found in paint systems. Traditional solvents have long been used for the degreasing of metal, glass or plastic work pieces in electroplating facilities, paint shops, and assembly plants, while new solvents have been introduced over the last few decades for specialized applications in the military, aerospace, biotechnology, and computer/semiconductor industries (Burgess, 1995).

Of the limited human studies of solvent exposure and semen quality, a number of them involve occupational exposure to broad classes of solvents as opposed to specific chemicals. For example, a study among 1,152 male partners in couples recruited from two infertility clinics in the Netherlands found that occupational exposure to aromatic solvents, estimated through questionnaires and a job-exposure matrix, was associated with increased risk of abnormal semen parameters (Tielemans *et al.,* 1999). Likewise, an association between solvent exposure and increased risk of abnormal sperm motility and morphology was reported in a study among 177 men that were interviewed about prior occupational exposures when reporting to an Argentina infertility clinic (Oliva *et al.,* 2001). A third study among men recruited from Canadian infertility clinics found a dose-dependent increased risk in abnormal sperm motility associated with moderate and high exposure to organic solvents (Cherry *et al.,* 2001).

Human studies of specific occupational solvent exposure and negative impacts on semen quality are limited but have been reviewed previously (Figa-Talamanca *et al.,* 2001; Sheiner *et al.,* 2003). Associations have been reported for ethylene glycol ethers (Shih *et al.,* 2000; Veulemans *et al.,* 1993; Ratcliffe *et al.,* 1989), trichloroethylene (Chia *et al.,* 1996), styrene (Kolstad *et al.,* 1999), benzene, toluene and xylene (Xiao *et al.,* 2001). However, because new chemical formulations continue to be introduced in industry to fit specific process requirements, reproductive toxicology and epidemiology data are not extensive for many solvents currently in use. Therefore it is possible that data implicating specific chemicals in reduced semen quality and male reproductive health will emerge in the future.

A recent example of emerging human evidence for reproductive toxicity of a specific solvent is that of 2-bromopropane. 2-bromopropane is sometimes used as an intermediate in the synthesis of pharmaceutical dyes and other organic chemicals (Boekelheide *et al.,* 2004), though in the US is found primarily as an impurity in 1-bromopropane that is used in spray adhesives or as a degreaser. In Asia, occupational exposure to 2-bromopropane is more prevalent since it is also used as a substitute for ozone-depleting chlorofluorocarbons (CFCs). A study of both male and female workers in a South Korean electronics factory that were exposed to 2-bromopropane reported compelling, though not conclusive, evidence of reproductive toxicity (Kim *et al.,* 1996). Of 8 male workers exposed to 2-bromopropane, 2 were azoospermic and 4 others had sperm concentration of less than 20 million/mL or less than 50% motile sperm. None of the 12 unexposed comparison workers from the same plant had abnormal semen measures. Follow-up studies in animals showed that 2-bromopropane led to impaired spermatogenesis (Ichihara *et al.,* 1997; Takeuchi *et al.,* 1997). Conversely, a subsequent study by Ichihara *et al.* (1999) among workers from a 2-bromopropane factory did not find an association between exposure and semen quality. However, the study had limited statistical power and exposure monitoring among the workers revealed few samples with exposure levels above those experienced by the reference group. Though the human and animal evidence was deemed sufficient by an expert panel convened by the National Toxicology Program to show male reproductive toxicity of 2-bromopropane (Boekelheide *et al.,* 2004), additional human studies investigating 2-bromopropane exposure and semen quality are needed.

4. Phthalates

The diesters of 1,2-benzenedicarboxylic acid (phthalic acid), commonly known as phthalates, are a group of man-made chemicals with a wide spectrum of industrial

applications. High molecular weight phthalates (e.g., di(2-ethylhexyl) phthalate [DEHP], di-isononyl phthalate [DiNP], di-n-octyl phthalate [DnOP]), are primarily used as plasticizers in the manufacture of flexible vinyl which, in turn, is used in consumer products, flooring and wall coverings, food contact applications, and medical devices (ATSDR, 1997; 2002; David *et al.,* 2001). Manufacturers use low molecular weight phthalates (e.g., diethyl phthalate [DEP] and dibutyl phthalate [DBP]) in personal-care products (e.g., perfumes, lotions, cosmetics), as solvents and plasticizers for cellulose acetate, and in making lacquers, varnishes, and coatings, including those used to provide timed releases in some pharmaceuticals (David *et al.,* 2001; ATSDR, 1995; 2001).

Because phthalates are widely used in many personal care and consumer products, the opportunity is high for non-occupational human exposure. However, to date, the proportional contribution from the various sources and routes of exposure to phthalates is unknown. Traditionally, ingestion has been considered an important route of exposure. Although phthalates have low volatility, they off-gas and are present in residential indoor air (Adibi *et al.,* 2003; Rudel *et al.,* 2003). Dermal contact (ATSDR, 1995; 1997; 2001; 2002) and parenteral exposure from medical devices containing phthalates may also contribute to exposure (ATSDR, 2002). Upon exposure, phthalates are rapidly metabolized and excreted in urine and feces (ATSDR, 1995; 1997; 2001; 2002). The most common biomonitoring approach for investigating human exposure to phthalates is the measurement of urinary concentrations of phthalate metabolites.

In the United States, the National Health Nutrition and Examination Survey (NHANES) is an ongoing survey, conducted by the National Center for Health Statistics at the Centers for Disease Control and Prevention (CDC), designed to collect data on the health and nutritional status of the civilian, noninstitutionalized U.S. population. The data estimates from NHANES, presented by age group, gender, and race/ethnicity, are probability-based, and hence, are representative of the US population. The recent third report from CDC (2005) on the NHANES survey confirmed that human exposure to selected phthalates (i.e., MEP, MBP, MBzP, and MEHP) is widespread among the US population.

As compared to the laboratory animal data on the reproductive toxicity of phthalates, the human data is very limited. All human studies to date were cross-sectional in design, adult exposure levels were measured and relationships with semen parameters were explored. In an early study, Murature *et al.* (1987) recruited 21 university students to explore the relationship between sperm concentration and DBP concentrations in the cellular fractions of ejaculates. The statistical analyses

performed were not traditional; that is, they did not treat the subjects as a single population. Instead, the authors assumed that there were two populations that differed in their ability to metabolize DBP. It is not entirely clear, but it seems that the two populations were defined by a visual inspection of DBP concentrations. Based on DBP concentrations, the subpopulations were defined as those with a lower ability to metabolize and those with a greater ability to metabolize DBP. In the subpopulation with a lower ability to metabolize DBP, there was an inverse relationship between sperm concentration and DBP (r = -0.4; slope of regression was -0.7). In the subpopulation with a greater ability to metabolize DBP, there was also an inverse correlation of -0.4 (slope of regression -0.6) between DBP and sperm concentration. The study was small and did not measure or adjust for potential confounders.

In India, Rozati *et al.* (2002) studied 21 infertile men with poor semen quality and 32 'control' men with normal semen parameters. Phthalate esters were measured in seminal plasma and the results were reported as the sum of a mixture of DMP, DEP, DBP, BBzP, DEHP, and DnOP. The concentration of phthalates was inversely correlated with sperm morphology (r = -0.77, p-value <0.001) and positively correlated with the percentage of single-stranded DNA in sperm (r = 0.86, p-value <0.001) assessed with the sperm nuclear chromatin condensation test. The concentration of phthalates was not correlated with ejaculate volume, sperm concentration, or motility. The authors measured total phthalate diesters and did not report results for individual phthalates. The results are noteworthy because they demonstrate the presence of phthalates in seminal plasma. However, because diesters were measured, sample contamination is a potential concern.

Duty *et al.* (2003a; 2003b; 2004) have published three manuscripts exploring the relationships between environmental exposure to phthalates and semen characteristics and sperm DNA damage. Study subjects consisted of male partners of subfertile couples that presented to an infertility clinic in Massachusetts, USA. At the time of the clinic visit, one sample of semen, blood and urine were collected from each subject. Computer-aided sperm analysis (CASA) was used to measure sperm concentration and motility, as well as motion parameters. Strict criteria were used to assess sperm morphology. Sperm DNA damage was assessed with the neutral comet assay. Because the Duty *et al.* (2004) study was ongoing, the number of subjects in each publication varies; however, there is overlap of subjects among the publications.

Among 168 men, they found dose-response relationships (after adjusting for age, abstinence time, and smoking status) between MBP and sperm motility (OR per tertile: 1.0, 1.8, 3.0; p for trend = 0.02) and sperm concentration (OR per tertile: 1.0,

1.4, 5.5; p for trend =0.07) (Duty *et al.,* 2003a). They also found a dose-response relationship between MBzP and sperm concentration (OR per tertile: 1.0, 1.4, 5.5; p for trend = 0.02). There was weak evidence of an association between MBP and sperm morphology, MBzP and sperm motility, and MMP and sperm morphology. Among 220 men, MBP, MBzP and MEHP had inverse associations, although not significant, with VSL (straight line velocity), VCL (curvilinear velocity) and LIN (linearity=VSL/VCL x 100) measured by CASA (Duty *et al.,* 2004). Unexpectedly, positive relationships were found between MEP and both VSL and VCL.

To quantify sperm DNA damage in samples analyzed using the neutral comet assay, Duty *et al.* (2003b) used VisComet image analysis software to measure comet extent, a measure of total comet length (micrometers), percent DNA in tail (tail %), a measure of the proportion of total DNA present in the comet tail, and tail distributed moment (TDM), an integrated measure of length and intensity (micrometers). In multiple regression models, after adjusting for age and smoking status, for an interquartile range (IQR) increase in MEP concentration the comet extent increased by 3.6 μm (95% CI: 0.74, 6.47) and TDM increased by 1.2 μm, (95% CI: -0.05, 2.38). There were no relationships between MBP, MBzP, MEHP and MMP and any comet assay parameters.

In a recently published study from Sweden, Jonsson *et al.* (2005) recruited 234 young Swedish men at the time of their medical conscript examination. Each man provided a single urine sample used to measure concentrations of MEP, MEHP, MBzP, MBP and phthalic acid. Semen quality was assessed using traditional semen parameters and sperm DNA integrity was measured by the sperm chromatin structure assay. Urinary phthalate levels were divided into quartiles and were used to calculate the mean difference and 95 per cent confidence interval between the lowest and highest quartiles. For MEHP, because 63 per cent had urinary concentrations below the detection limit (15 ng/mL), they compared these men with the 18 per cent of men who had the highest concentrations of MEHP. Because multivariate adjusted and unadjusted results differed by less than 15 per cent, potential confounders, such as abstinence time and smoking status, were not kept in the models.

In contrast to the US study, there were no relationships of MBP or MBzP with any of the reproductive markers. MEHP was also not associated with any of the reproductive markers. Men in the highest quartile for MEP had fewer motile sperm (mean difference was 8.8%; 95% confidence interval 0.8, 17) and more immotile sperm (8.9%; 0.3, 18) than men in the lowest MEP quartile. Contrary to their hypothesis, phthalic acid was associated with improved function as measured by more motile sperm and fewer immotile sperm. Phthalic acid is a non-specific marker of phthalate exposure, formed as the result of the hydrolysis of any of the phthalates

measured. Interactions between urinary phthalate levels and PCB 153 (measured previously in serum samples from these men) were assessed by including an interaction term in the models. There was no evidence of multiplicative interactions between PCB 153 and any of the phthalates with the reproductive markers (data was not shown). This is in contrast to a previous study by Hauser *et al.* (2005), where they found interactions of MBP and MBzP with PCB 153 in relation to sperm motility.

Although the Swedish study had similarities in design and execution to the US study, there were important differences. The study population in the Swedish study consisted of young men (median age 18 years, range 18-21 years) that were undergoing a medical examination before military service. Since approximately 95 per cent of young men in Sweden undergo the conscript examination, these young men reflected the general population of young Swedish males. In contrast, in the US study, the median age of the men recruited from an infertility clinic was 35.5 years and ranged from 22 to 54 years. None of the men from the infertility clinic were 21 years of age or younger. The recruitment of men from an infertility clinic as compared to young men from the general population may account for some of the differences in results between studies. For instance, it is unclear whether men presenting to an infertility clinic are more 'susceptible' to reproductive toxicants, including phthalates, than men from the general population. Furthermore, it is also unclear whether middle-aged men, as compared to young men, are more susceptible to reproductive toxicants because of an age related response to the toxicant.

Although only 14 per cent of the young Swedish men, as compared to 65 per cent of men in the present study, agreed to participate, it is unlikely that the young Swedish men did so differentially in relation to reproductive function and phthalate levels. Therefore, selection bias as a result of the low participation rate is unlikely in the Swedish study.

Despite similarities in urinary concentrations of the phthalate monoesters across studies, the analytical methods differed between the Swedish and US study. The detection limits for MEP, MBP, MBzP, and MEHP in the Swedish study were 30, 15, 7, 15 ng/mL, many fold higher than the detection limits (~ 1 ng/mL) in the US study. In addition, the precision from comparisons of duplicate analysis on different days was low in the Swedish study and likely due to the lack of isotope-labelled standards for the phthalate monoesters measured. In the US study, the phthalate monoesters were measured using isotope-dilution high performance liquid chromatography tandem mass spectrometry (Blount *et al.,* 2000; Silva *et al.,* 2003; 2004). The isotope dilution method is precise, with relative standard deviations of less than 15 per cent from replicate measurements. The higher limits of detection and lower analytical precision

in the Swedish study may contribute to measurement error of urinary phthalate levels and may result in bias to the null hypothesis. However, by categorizing the phthalate levels into quartiles for the statistical analysis, some of the measurement error resulting from the analytical imprecision and low detection limits may be minimized. The Swedish study used urinary creatinine to adjust for urine dilution as compared to specific gravity in the US study. Based on the medians in the tables from the Swedish study, the creatinine adjusts values were quite different from the unadjusted values. In contrast, in the US study, medians between SG-adjusted and unadjusted values were not markedly different.

The statistical methods used for the data analysis also differed between studies and may partially account for the differences in results. In the US study, multivariate logistic regression with categorized semen parameters was used as the primary outcome. Men with all three semen parameters above the reference range were used as comparison subjects in these analyses. In contrast, in the Swedish study, for the primary analysis, semen parameters were used as a continuous measurement and mean differences between men in the highest and lowest phthalate quartiles were calculated. In addition, logistic regression analyses were performed, although the results of these analyses, reported to be consistent with their primary analyses, were not shown. However, it is unclear whether the comparison group in the logistic regression analyses included only men with all three semen parameters above the reference range. If not, dilution of associations between phthalates and semen parameters may occur since the comparison group does not consist of a homogenous group of men with normal semen parameters. For instance, dilution of the associations between sperm concentration and phthalate monoesters may occur if phthalates alter both sperm concentration and motility.

In conclusion, the epidemiologic data on the relationship between semen quality and phthalate exposure remains limited and inconsistent. Although the two large recent studies by Duty *et al. (2004)* and Jonsson *et al. (2005)* had many similarities, important differences existed. The US study recruited older men from an infertility clinic while the Swedish study recruited young men from the general population. It is currently unclear whether these differences in age and recruitment source may partially account for the inconsistent results across studies, especially for MBP and MBzP. Additional studies are critically needed to help elucidate possible explanations for differences across studies, and most importantly to address whether phthalate exposure alter semen quality.

Table 2. Summary table of epidemiologic studies (in chronological order) on the relationship between phthalates and semen quality

Author, country	Study population	Exposure	Results	Comments
Murature *et al.,* 1987 US	21 young men	DBP in cellular fractions of ejaculates	In men with 'low ability to metabolize DBP', inverse relationship between sperm concentration and DBP (r=-0.4; slope of regression was -0.7). In the 'men with a greater ability to metabolize DBP', there was also an inverse correlation of -0.4 (slope of regression -0.6) between DBP and sperm concentration	Small sample size, no adjustment for confounders
Rozati *et al.,* 2002 India	53 men (21 infertile and 32 controls)	Seminal plasma levels of phthalates (DBP, BBzP, DEHP, DnOP)	Sum of phthalates was inversely correlated with sperm morphology (r=-0.77, p-value < 0.001) and positively correlated with the percentage of single-stranded DNA in sperm (r=0.86, p-value <0.001) assessed with the sperm nuclear chromatin condensation test. The concentration of phthalates was not correlated with ejaculate volume, sperm concentration, or motility.	Measured total phthalate diesters, concern with contamination
Duty *et al.,* 2003a, 2004 US	168 men from an infertility clinic (semen parameter), 220 men (CASA results)	Urinary levels of phthalate metabolites (MBP, MBzP, MEP, MEHP, MMP)	Dose-response relationships (after adjusting for age, abstinence time, and smoking status) between MBP and sperm motility (OR per tertile: 1.0, 1.8, 3.0; p for trend = 0.02) and sperm concentration (OR per tertile: 1.0, 1.4, 5.5; p for trend =0.07). Dose-response relationship between MBzP and sperm concentration (OR per tertile: 1.0, 1.4, 5.5; p for trend = 0.02). MBP, MBzP and MEHP had inverse non-significant associations with VSL (straight line velocity), VCL (curvilinear velocity) and LIN (linearity=VSL/VCL x 100).	Confounders considered: age, BMI, abstinence time, smoking status, race

Table 2. continued.

Author, country	Study population	Exposure	Results	Comments
Duty *et al.,* 2003b US	168 men from an infertility clinic	Urinary levels of phthalate metabolites (MBP, MBzP, MEP, MEHP, MMP)	After adjusting for age and smoking status, for an interquartile range increase in MEP concentration the comet extent increased by 3.6 μm (95% CI: 0.74, 6.47) and tail distributed moment increased by 1.2 μm, (95% CI: -0.05, 2.38). There were no relationships between MBP, MBzP, MEHP and MMP and any comet assay parameters.	Confounders considered: age, BMI, abstinence time, smoking status, race
Jonsson *et al.,* 2005 Sweden	234 young men	Urinary levels of MEP, MEHP, MBzP, MBP and phthalic acid	No relationships of MBP, MBzP, or MEHP with any of the semen parameters. The highest quartile for MEP had fewer motile sperm (mean difference was 8.8%, 95% CI: 0.8, 17) and more immotile sperm (8.9%, 95% CI: 0.3, 18). Phthalic acid was associated with improved function as measured by more motile sperm and fewer immotile sperm.	Confounders considered: abstinence time, smoking status.

Abbreviations: di(2-ethylhexyl) phthalate [DEHP]; monoethylhexyl phthalate [MEHP]; di-isononyl phthalate [DiNP]; di-n-octyl phthalate [DnOP]); diethyl phthalate [DEP]; monoethyl phthalate [MEP]; butylbenzyl phthalate [BBzP]; dibutyl phthalate [DBP]; monobutyl phthalate [MBP]; monobenzyl phthalate [MBzP].

5. Polychlorinated biphenyls and dichlorodiphenyl trichloroethane (DDT)

Polychlorinated biphenyls (PCBs) are a class of synthetic, persistent, lipophilic, halogenated aromatic compounds that were widely used in industrial and consumer products for decades before their production was banned in the late 1970's. PCBs were used in cutting oils, lubricants and as electrical insulators. As a result of their extensive use and persistence, PCBs remain ubiquitous environmental contaminants. They are distributed worldwide and have been measured in air, water, aquatic and marine sediments, fish, and wildlife (De Voogt and Brinkman, 1989). Furthermore, they are biologically concentrated and stored in human adipose tissue. The general

population is exposed primarily through ingestion of contaminated foods (e.g., fish, meat, and dairy products), as PCBs can bioaccumulate up the food chain. However, exposure may also occur through dermal contact (soil and house dust) and inhalation (indoor air in residential buildings and workplaces, as well as outdoor air). For example, in the 1960s and 1970s, PCBs were used in sealants for commercial building construction, and high levels of PCBs (up to 36,000 ppm) have been found to remain in the caulking of some public buildings that may lead to contamination of indoor air and dust (Herrick *et al.,* 2004). As a result of their persistence and ubiquity, measurable levels of serum PCBs are found in the majority of the U.S. general population (Longnecker *et al.,* 1997). Serum levels of PCBs are an integrated measure of internal dose, reflecting exposure from all sources over the previous years; depending on the congener, the half-life of PCBs in the blood ranges from one to ten or more years (Brown, 1994; Phillips *et al.,* 1989).

5.1. Studies on environmental exposure

In an early study on PCBs and semen quality, Bush *et al.* (1986) studied semen samples from fertile men (n = 33), men with oligospermia (n = 50) or azoospermia (n = 50) and men status post-vasectomy (n = 25). The average (SD) age of the men from these groups was 33 (7), 32 (4), 33 (5), 38 (7) years, respectively. The seminal concentrations of PCBs 153, 138, and 118 were inversely related to sperm motility only among samples with a sperm count less than 20 million/mL. The authors caution against over interpreting these associations because they were found only among a subset of subjects.

In the Netherlands, Dallinga *et al.* (2002) studied the relationship between PCBs and semen quality among men that were partners in couples visiting an infertility treatment center. They identified two groups of men, those men with good semen quality (n = 31) and men with very poor semen quality (n = 34) based on three semen samples. Progressive motile sperm concentration was used to make the classification. A Makler counting chamber was used to measure sperm concentration and motility and strict criteria were used for sperm morphology. Blood and semen were analyzed for PCB 118, 138, 153, and 180 and their hydroxylated metabolites. The mean (SD) non-lipid adjusted levels of PCB 153 were 0.41 (0.22) ng/g blood. Assuming that serum percent lipid is approximately 0.5 per cent, the estimated lipid adjusted concentration would be 82 ng/g lipids. Contrary to expectations, the sum of PCBs in seminal plasma of men with good semen quality was higher than among men with poor semen quality (0.071 ng/mL and 0.022 ng/mL seminal plasma, respectively, p-value = 0.06). However, within the group of men with good semen

quality, there were inverse associations between serum levels of sum of PCB metabolites and sperm count (p-value = 0.04) and progressive motile sperm concentration (p-value = 0.02). There were also negative non-significant corresponding associations in the men with poor semen quality. Because associations with semen quality were found for PCB metabolites and not the parent PCBs, these results suggested that the PCB metabolites were the biologically active compounds.

Richthoff *et al.* (2003) conducted a study on the relationship between PCB 153 and semen parameters among 305 young men undergoing a conscript examination for military service. The men ranged in age from 18 to 21 years with a median age of 18.1 years. PCB 153 levels were considered representative of background environmental levels for men from Southern Sweden; the median was 65 ng/g lipid with a range from 23 to 250 ng/g lipid. PCB 153 is a good biomarker of exposure to total PCBs and toxic equivalents (TEQ) (Gladen *et al.,* 1999; Grimvall *et al.,* 1997). Semen samples were analyzed according to WHO recommendations (1999). Sperm concentration was assessed by a modified Neubauer chamber. Sperm motility (categories A, B, C, and D) was assessed according to WHO recommendations and the percentage of motile sperm was assessed by use of CRISMAS computer-aided sperm motility analyzer (CASA) with a Makler chamber. The following confounders were considered for inclusion in the models: BMI, abstinence period, and smoking status. There were significant inverse associations between PCB 153 and percent motile sperm; a 10ng/g lipid increase in PCB 153 was associated with a 1.0 per cent decline in percent CASA motile sperm (95% CI: -2.0, -0.13). The association between PCB 153 and conventional sperm motility was slightly weaker. There were no associations between PCB 153 and sperm concentration or total sperm count. The study was relatively large and well conducted. Although the participation rate was very low, only 13.5 per cent of eligible subjects agreed to participate, it is unlikely that this would introduce bias since young men are likely to be unaware of their fertility or exposure levels.

Rozati *et al.* (2002) measured PCBs in seminal plasma and explored relationships with semen parameters among men in India. Details of the study are provided above in the phthalate section. PCBs were detected in the seminal plasma of infertile men but not controls. They reported a negative correlation between seminal plasma PCB levels and total progressive motility (r = -0.5) and a positive correlation with percentage of single-stranded DNA in sperm (r = 0.6). No correlations were found between PCBs and sperm count, rapid progressive motility or normal morphology. The authors reported results for total PCBs and not for individual congeners.

Potential confounders were considered in the method section but no adjustments were made.

Rignell-Hydbom *et al.* (2005; 2004) reported on the associations between PCBs and p,p'-DDE with semen parameters and sperm chromatin integrity. Swedish fishermen from the east and west coasts were studied. 195 Swedish fishermen (median age 50.6 years, ranged from 24-65 years) participated in the semen quality study and 176 of these men had semen samples analyzed for sperm chromatin integrity, assessed by sperm chromatin structure assay (SCSA). The median serum levels of PCB 153 and p,p'-DDE (dichlorodiphenyl-dichloroethene) were 193 ng/g lipid (ranged from 39 to 1,460) and 240 ng/g lipid (ranged from 334 to 2,251), respectively. When PCB 153 was categorized into quintiles, the highest quintile had decreased sperm motility compared with men in the lowest quintile. The age adjusted mean difference was 9.9 per cent (95% confidence interval -1.0 to 21%, p-value = 0.08). There were no consistent associations of PCB 153 with sperm concentration. Although p,p'-DDE was inversely associated with sperm motility, when age was included in the models the association became weaker and non-significant. Among men with SCSA results, there was a univariate association between log-transformed PCB 153 and the percentage of sperm showing DNA fragmentation (%DFI), r = 0.27, p-value < 0.001). When age was adjusted for the association was no longer significant (p-value = 0.28). However, in age adjusted analyses in which PCB 153 was divided into quintiles, the lowest quintile had significantly lower %DFI (p-values < 0.006). Although PCB 153 and p,p'-DDE were highly correlated (r = 0.8), the relationship between p,p'-DDE and %DFI was less consistent (p-value = 0.1). Interestingly, there was a moderate correlation between sperm motility and %DFI, which suggests that PCB 153 may alter both through a common pathway or that alteration of one may in turn affect the other parameter.

Hauser and colleagues (2003a) conducted a study on 212 male partners of sub-fertile couples visiting an infertility clinic in Massachusetts, US. Sperm concentration and motility were assessed with CASA and morphology with the strict criteria. The mean (SD) age was 36.0 (5.4) years. 57 PCB congeners were measured, and included PCB 118, 138, 153 and 180. The median levels for PCB 153 and p,p'-DDE were 42 ng/g lipid (range 9.3 to 361) and 222 ng/g lipid (range 64 to 8912), respectively. Multivariate logistic regression analyses were used in which semen parameters were dichotomized based on WHO reference values (1999). The comparison groups for each analysis were defined as men with all three semen parameters above reference values. There were significant dose-response relationships (odds ratio per tertile adjusted for age, abstinence time, and smoking status) between PCB 138 and below reference sperm motility (1.00, 1.68, 2.35,

respectively; p-value for trend 0.04) and sperm morphology (1.00, 1.36, 2.53; p-value=0.04). Associations between semen parameters and PCB 153 were not consistent. DDE showed a weak non-significant relationship with sperm motility. Hauser *et al.* (2003b) also studied the relationship between PCBs and p,p'-DDE with DNA integrity in sperm using the neutral comet assay. They did not find any strong or consistent associations between any of the PCB congeners and p,p'-DDE with measures of DNA integrity.

Although the pesticide DDT was banned for use in most industrialized countries, it is currently used for malaria control in several countries. Ayotte *et al.* (2001) reported on the association between p,p'-DDE, a major biologically persistent metabolite of DDT, and semen quality in 24 young men from Chiapas, Mexico. The men, 16 to 28 years of age, were non-occupationally exposed to DDT. The mean concentration of p,p'-DDE was 77.9 mg/kg (range, 17 to 177), a value several hundred fold higher than levels in men from other countries, such as the US and Canada, where DDT was not recently used. p,p'-DDE was inversely correlated with both semen volume (r = -0.47) and sperm count (r = 0.4). Although the study was small and did not control for potential confounders, the results are intriguing and worthy of replication in other cohorts.

5.2. High PCB exposure studies

Guo *et al.* (2000) studied the relationship between semen quality and prenatal exposure to PCBs and PCDFs after the poisoning episode in Taiwan in 1979 in which PCB contaminated rice oil was ingested. In 1998, 12 men pre-natally exposed to contaminated rice oil and 23 healthy unexposed subjects of comparable age provided a semen sample. The unexposed men had no unusual chemical exposure and were recruited from a local high school. Sperm motility was assessed using a Makler chamber and also by CASA. Morphology was assessed using the WHO guidelines. The mean (SD) age of the exposed men was 17.3 (1.2) years and 17.6 (1.0) for the unexposed men. The proportion of sperm with abnormal morphology was increased in the exposed men (37.5% as compared to 25.9% for unexposed men). In the exposed men the percentage of motile sperm (35.1% compared to 57.1% in unexposed men) and rapidly motile sperm (25.5% compared to 42.4 % in unexposed men) were reduced. Several of the CASA parameters were reduced in the exposed men, in particular, average path velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL). Sperm from exposed men had reduced hamster oocyte penetration as compared to unexposed men. This small study provided the opportunity to explore high pre-natal exposure to PCBs and PCDFs.

In another study on men from the Taiwan PCB poisoning, Hsu *et al.* (2003) studied the relationship between semen quality and levels of PCBs among men that consumed contaminated rice oil some twenty years earlier. They identified 40 exposed men and 28 unexposed men that were matched using an address registry. Mean age of exposed (37.9 years) and unexposed (40.4) were similar. Exposed men had a higher percentage of sperm with abnormal morphology (27.5% compared to 23.3%) and a higher oligospermia rate (9% compared to 1%). The ability of sperm to penetrate the hamster oocyte was reduced in exposed men. The results of this small study provide evidence of adverse effects of exposure to PCBs and PCDFs among men exposed 20 years earlier to the contaminated rice oil.

Table 3. Summary table of epidemiologic studies (in chronological order) on the relationship of polychlorinated biphenyls and p,p-DDE with semen quality

Author	Study population	Exposure	Results	Comments
Bush *et al.,* 1986	33 fertile, 50 subfertile, 50 infertile, and 25 post-vasectomy men	Seminal plasma levels of PCBs and p,p'-DDE	PCB 153, 138, and 118 were inversely related to sperm motility only among samples with a sperm count less than 20 million/mL. No associations of semen parameters with p,p'-DDE.	Association found only among a subset of men.
Rozati *et al.,* 2002	53 men from India (21 infertile and 32 controls)	Seminal plasma levels of PCBs	PCBs detected in the seminal plasma of infertile men but not controls. Negative correlation between PCBs and total progressive motility (r=-0.5), and positive correlation with percentage of single-stranded DNA in sperm (r=0.6). No correlations with sperm count, rapid progressive motility or normal morphology.	Data on individual PCB congeners not presented. No statistical adjustment for potential confounders.

Table 3. continued.

Author	Study population	Exposure	Results	Comments
Dallinga *et al.,* 2002	65 Dutch men from an infertility clinic	Serum and semen levels of PCB 118, 138, 153, 180 and their metabolites	Seminal plasma PCB levels among men with good semen quality were higher than among men with poor semen quality (p-value =0.06). In men with good semen quality, there were inverse associations between serum levels of sum of PCB metabolites and sperm count (p-value = 0.04) and progressive motile sperm concentration (p-value = 0.02). There were also negative non-significant corresponding associations in men with poor semen quality.	Confounders considered: age and smoking status. Measured PCB metabolites.
Richthoff *et al.,* 2003	305 Swedish young men	Serum levels of PCB 153	Inverse association between PCB 153 and percent motile sperm (10 ng/g lipid increase in PCB153 associated with a 1.0% decline in percent CASA motile sperm (95% CI: -2.0, -0.13)). No association of PCB 153 with sperm concentration.	Confounders considered: BMI, abstinence period, smoking status.
Hauser *et al.,* 2003	212 US men from an infertility clinic	Serum levels of PCBs and p,p'-DDE	Dose-response relationships (odds ratio per tertile adjusted for age, abstinence time, and smoking status) between PCB 138 and below reference sperm motility (1.00, 1.68, 2.35, respectively; p-value for trend 0.04) and sperm morphology (1.00, 1.36, 2.53; p-value=0.04). DDE had a non-significant association with sperm motility.	Confounders considered: BMI, age, abstinence period, smoking status.

Table 3. continued.

Author	Study population	Exposure	Results	Comments
Hauser *et al.,* 2003	212 US men from an infertility clinic	Serum levels of PCBs and p,p'-DDE	No associations between any of the comet assay parameters and PCBs or DDE.	Confounders considered: age, smoking status.
Rignell-Hydbom *et al.,* 2004	195 Swedish fishermen	Serum levels of PCB 153 and p,p'-DDE	The highest PCB 153 quintile had decreased sperm motility as compared with men in the lowest quintile. The age adjusted mean difference was 9.9% (95% confidence interval -1.0 to 21%, p-value=0.08). No significant associations of p,p-DDE with semen parameters.	Confounders considered: age, smoking status, abstinence time, BMI, reproductive hormones.
Rignell-Hydbom *et al.,* 2005	176 Swedish fishermen	Serum levels of PCB 153 and p,p'-DDE	Age adjusted analyses, the lowest PCB quintile had significantly lower %DFI than the other quintiles (p-values < 0.006). Non-significant relationship between p,p'-DDE and %DFI (p-value = 0.1).	Confounders considered: age, smoking status, abstinence time, BMI, reproductive hormones.
High Exposure Studies				
Guo *et al.,* 2000	35 young men from Taiwan (12 pre-natally exposed to contaminated rice oil, 23 unexposed men)	Maternal ingestion (yes/no) of rice oil contaminated with PCBs and PCDFs	Increased abnormal morphology in exposed men (37.5%) as compared to unexposed men (25.9%) for unexposed men. Exposed men had decreased percentage of motile sperm (35.1% compared to 57.1% in unexposed men) and rapidly motile sperm (25.5% compared to 42.4 % in unexposed men). Reduced hamster oocyte penetration in exposed men.	Age and % smokers in exposed and unexposed groups were similar. No statistical adjustment for confounders.

Table 3. continued.

Author	Study population	Exposure	Results	Comments
Hsu *et al.,* 2003	68 men from Taiwan (40 exposed to contaminated rice oil and 28 unexposed)	Ingestion (yes/no) of rice oil contaminated with PCBs and PCDFs	Exposed men had higher percentage of sperm with abnormal morphology (27.5%) compared to unexposed men (23.3%), and a higher oligospermia rate (9% compared to 1%, respectively). Ability of sperm to penetrate the hamster oocyte was reduced in exposed men.	Age and % smokers in exposed and unexposed groups were similar. No statistical adjustment for confounders

Abbreviations: body mass index [BMI], polychlorinated biphenyls [PCBs], polychlorinated dibenzofurans [PCDFs]

The data on the relationship between PCBs and semen quality support an inverse association of PCBs with reduced semen quality, specifically reduced sperm motility. The associations found were generally consistent across studies performed in different countries (India, the Netherlands, Taiwan, Sweden, and US) that used different methods to measure semen quality and PCBs. Furthermore, associations were consistently found despite a range of PCB levels, that is there did not appear to be a threshold. The PCB levels in these studies ranged from low background levels (Hauser *et al.,* 2003a; 2003b; Richthoff *et al.* 2003; Dallinga *et al.,* 2002), to high background levels due to consumption of contaminated fish (Rignell-Hydbom *et al.,* 2004; 2005a; 2005b), to even higher exposure levels due to ingestion of contaminated rice oil (Guo *et al.,* 2000; Hsu *et al.,* 2003). Although the data across studies generally support a relationship between PCBs and poor semen quality, there are possible alternative explanations. One potential alternative explanation is that PCBs are a surrogate for exposure to other environmental factors that may predict semen quality. Although this is possible, there is currently no evidence identifying potential alternative exposures. Another explanation is that there may be confounding of the associations by some currently unrecognized or unmeasured confounders. Although possible, this is also unlikely because the more recent studies considered important potential confounders and the results were consistent across studies suggesting that it is unlikely that there is a strong unmeasured confounder. In conclusion, although PCBs are no longer used, this data, along with ongoing human exposure, albeit at lower levels than several decades ago, raise concerns regarding altered human fertility due to adverse affects on semen quality.

6. Emerging compounds

In addition to the chemicals discussed in this chapter, there are other classes of chemicals that require further study as to their relation with human semen quality. These chemicals include alkylphenols, such as 4-nonylphenol, bisphenol A (BPA) and fluorinated organic compounds such as perfluorooctane octanoate (PFOA) and perfluorooctane sulfonate (PFOS). Alkylphenols are used as surface active agents in cleaning/washing agents, paints, and cosmetics, while BPA is used in the manufacture of polycarbonate plastics and epoxy resins. The perfluorinated compounds are used to make fabrics stain-resistant/water repellant and in coatings on cookware and other products. Although human exposure to these chemicals has been demonstrated, the health effects data in humans remains severely limited.

7. Conclusions

This chapter presents an up-to-date summary of recently published human studies on the relationship between semen quality and exposure to several classes of environmental chemicals. The classes of chemicals included in this chapter represent a number of contemporary and widely used compounds such as insecticides and phthalates. In addition, although PCBs were banned several decades ago, they are also discussed because over the last five years many well-designed epidemiologic studies have been published.

Recent studies, along with several earlier studies, suggested that exposure to specific non-persistent insecticides may be associated with poorer semen quality. Two occupational studies of workers exposed to the insecticide carbaryl reported suggestive evidence of lowered sperm concentration and morphology, while a more recent study of environmental exposure to carbaryl found an association with reduced sperm concentration and motility. Recent evidence also suggested a relationship between occupational and environmental exposure to some organophosphorus insecticides and reduced sperm concentration and motility.

Solvents, like pesticides, represent a large number of chemicals with varying reproductive toxicities. Adverse effects on semen quality associated with exposure to some solvents, such as ethylene glycol ethers, were recognized, while potential adverse effects on the human reproductive system for many other solvents were not well-studied. As new chemicals are introduced and more studies are conducted, evidence of effects on semen quality in relation to solvent exposure may emerge as in the recent case of 2-bromopropane.

For phthalates, a widely used class of chemicals, there was suggestive but inconsistent evidence for an inverse relationship with semen quality, specifically between monobutyl and monobenzyl phthalate and sperm motility and concentration. For exposure to PCBs, there was consistent evidence of associations with poorer sperm motility. The inverse associations between PCBs and sperm motility were consistent across studies conducted in several different countries (India, the Netherlands, Taiwan, Sweden, and US) despite the use of different methods to measure semen quality and PCBs. Furthermore, the inverse associations were also consistently found despite a range of PCB levels, that is there did not appear to be a threshold.

Although the epidemiologic data on these historic, contemporary and emerging environmental contaminants suggest that there may be associations with altered semen quality, the quantity and quality of the data available for the different types of compounds varied. For example, though there are hundreds of different pesticides currently in use worldwide, limited human data existed on male reproductive endpoints for only a select few. Also, for some of these chemicals, such as phthalates, the data across studies was not entirely consistent. For instance, one study found associations of semen quality with monobutyl phthalate and monobenzyl phthalate while another large epidemiologic study did not. The limited human data, and in certain instances inconsistent data across studies, highlight the need for further epidemiological research on these classes of chemicals.

A future challenge to understanding the relationship between these chemicals and semen quality includes the changes in exposure levels among populations over time due to the ever-changing patterns of production and use of these compounds. Another challenge is to understand how simultaneous coexposures to these chemicals may affect semen quality. It is well known that humans are exposed to all of these compounds simultaneously, as well as to many other chemicals. However, there is limited data on the interactions between chemicals within a class or across classes of chemicals. Chemicals may interact additively or multiplicatively, or antagonistically. The human health risks of exposure to chemical mixtures is very understudied. Despite these challenges, evolving and innovative technologies designed to improve the assessment of human exposure and male reproductive health endpoints should provide enhanced opportunities for improving our understanding of the relationship between environmental chemicals and semen quality. Innovations include improved biomarkers of exposure and more sophisticated statistical methods that deal with multiple exposures simultaneously.

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ENVIRONMENT AND FERTILITY

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Summary

The reproductive process is markedly affected by the environment. Toxic substances in the physical ecosystem, along with biological, social, cultural, and economical aspects of exposed populations, are factors that interact with each other, making the study of the effects of environmental variables on reproduction quite complex. Despite the difficulties in approaching the problem, the amount of available evidence supporting the concept that environmental contamination represents a risk factor to fertility steadily increases. In some circumstances, it is possible to identify precisely the toxic agent responsible for fertility impairment, as in the case of heavy metals. In other cases, the adverse effects are the result of exposure to complex mixtures, such as wood stoves, automotive and industrial emissions, and several classes of endocrine disrupting substances. The mechanisms responsible for environmental-dependent impairment of fertility are not fully identified, but some basic pathways can be presented: direct toxicity, induction of mutations, oxidative stress and endocrine disruption. Although in some cases the risk excess is small, there are toxic agents – such as air pollutants and contaminants of public water – affecting millions of people, increasing the risk attributed to these diffuse environmental threats.

1. Introduction

The reproductive process is markedly affected by the environment. Toxic substances in the physical ecosystem, biological, social, cultural, and economical aspects of the exposed populations are interacting factors, making the study of effects of

P. Nicolopoulou-Stamati et al., (eds.), Reproductive Health and the Environment, 57–71.

environmental variables on reproduction quite complex. Bad working conditions, poor sanitation and nutrition, high prevalence of infectious diseases, and limited access to vaccination and medical care, are markers of a poor socio-economic environment that is associated with an increased exposure to toxic substances. Old vehicles, low technology in industrial and agricultural processes are more frequent in developing economies, as well as in the poorest segments of richer countries. These considerations are the basis for the concept of environmental racism or environmental injustice (Silbergeld and Patrick, 2005). However, the adverse effects of pollution are not restricted to developing regions. Even in developed economies pollution is abundant. Pollutants that may affect reproduction include air emissions from power plants and vehicles, endocrine disrupting agents and water disinfection by products (Table 4).

Table 4. Classes of main environmental pollutants that potentially interfere with human reproduction

Tobacco smoke	Emissions from biomass burning
Industrial emissions	Emissions from waste incinerators
Automotive emissions	Emissions from power plants
Metals	Radiation
Pesticides	Food contaminants
Water disinfection by products	Exogenous hormones

In an attempt to address the relationship between environment and reproduction, some remarks should be made. Firstly, this paper will focus on the aspects related to toxins in the physical environment. It will not deal with the social, economic and environmental conditions. Secondly, pollution caused by tobacco smoke and its effects on reproduction will not be addressed in the present chapter, as this problem has been extensively described. Even with the above restrictions, the list of environmental substances capable of interfering with human reproduction and the number of people at risk indicate that this represents a significant health issue.

2. Abnormalities in male and female gamete quality, production and regulation

Several environmental contaminants have been associated with gamete alterations in males and females. They include heavy metals, chemical agents widely used in

agriculture, industry, phyto- and xeno-hormones, and endocrine disrupting substances (Sinawat, 2000). Several end-points characterize the impacts of environmental variables on fertility.

2.1. Sperm quality and quantity

Sperm quality decreased in the last decades (Nelson and Bunge, 1974; Bendvold, 1989; Bendvold *et al.,* 1991; Bostofte *et al.,* 1983; James, 1980; Leto and Frensilli, 1981; Menkveld *et al.,* 1986; Osegbe *et al.,* 1986; Osser *et al.,* 1984; Smith *et al.,* 1978). It is not clear however whether these changes reflect a selection bias or a true biological phenomenon (Multigner and Oliva, 2002). A meta-analysis of 61 publications (Carlsen *et al.,* 1992) reported that the volume of ejaculate and sperm cell counts were reduced by 26% and 50% respectively during the period from 1938 to 1990. The study provoked a lot of debate (Becker and Berhane, 1997; Bromwich *et al.,* 1994; Farrow, 1994; Fisch and Goluboff, 1996; Olsen *et al.,* 1995; Saidi *et al.,* 1999; Swan *et al.,* 1997) because of the different methods and the possible confounding variables present in the studies included in the analysis. More controlled studies, performed in different centers found significant reductions in sperm parameters over time (Adamopoulos *et al.,* 1996; Auger *et al.,* 1995; Bilotta *et al.,* 1999; Bonde *et al.,* 1998; Irvine *et al.,* 1996; Menchini-Fabris *et al.,* 1996; Zorn *et al.,* 1999), whereas no change was reported by other authors (Acacio *et al.,* 2000; Bujan *et al.,* 1996; Paulsen *et al.,* 1996; Rasmussen *et al.,* 1997; Vierula *et al.,* 1996). Despite the fact that the debate on a secular trend of sperm quality is still open, it is tempting to point to environmental factors as a possible cause of changes in sperm quality.

2.2. Testicular function

Testes are the target organ of several contaminants of the environment. Metals widely present in ambient particles, such as cadmium, chromium and lead, accumulate in the testis and promotes tissue injury (Danielsson *et al.,* 1984; Rodamilans *et al.,* 1988). Organic substances, such as polychlorinated biphenyls, accumulate not only in sperm, but also follicular fluid (Schelebusch *et al.,* 1989). Pesticides, such as chlordecone and dibromochloropropane, are associated with reduced sperm counts and low fertility (Hruska *et al.,* 2000; Longnecker *et al.,* 1997).

Air pollution is a complex mixture of contaminants. Exposure to air pollutants, makes it difficult to determine one single agent that is responsible for causing injury to the testes. Men living in industrial areas are reported to show impaired sperm quality and endocrine alterations, due to pollution of endocrine disrupting substances (Dhooge *et al.,* 2001). Heritable mutation frequency increases at tandem-repeat DNA loci, as observed in mice exposed *in situ* to polluted air near a steel mill: the effect was due to an increase in mutations inherited through the paternal germline (Sommers *et al.,* 2002; 2004). This indicates that the testes function is not only affected by high levels of pollution in the working environment, but also by ambient levels of air pollution. Moreover, the system showed that rodents can be used to monitor the environmental effects on the testes function. (Tannenbaum *et al.,* 2003).

2.3. Decreased reproductivity in females

Also, female reproductive organs are adversely affected by environmental pollutants. One mechanism of reproductive toxicity is the depletion of ovarian follicles after exposure to chemicals. This results either in an earlier age of onset of menopause, or in a disruption of the ovulation cycle (Borgeest *et al.,* 2002; Mayer *et al.,* 2002). In addition, the fertilization rate of the oocytes may be impaired by toxic chemicals such as trichloroethylene or tetrachloroethylene (Berger and Horner, 2003).

The hormone balance has a pivotal role in human fertility. It regulates both the production of gametes in adequate quantity and the timing of the ovulation cycle. Several categories of hormone disrupting substances exist. They vary in potency, persistence in the environment, and capacity to bioaccumulate. Examples include synthetic hormonal drugs, dioxins (associated with waste incineration and other industrial incineration activities), polychlorinated biphenyls (plastic and electric industry), alkylphenols and phthalates (pesticides and plastics), and metals (cadmium and lead). These substances may impair the hypothalamic-pituitary-gonadal control, and affect ovulation (Silbergeld and Patrick, 2005). Other mechanisms acting as quantitative and qualitative alterations of the gametes, and affecting the control of the ovulation cycle, may be responsible for fertility impairment.

Table 5 provides a summary of the mechanisms and agents responsible for adverse impacts on gamete quality, production and regulation.

Table 5. Examples of toxic environmental contaminants exhibiting detrimental effects on gamete quality, production and regulation

Type of Contaminant	Main Source	Plausible Mechanism
Metals	Automotive	Oxidative stress
	Industry	Endocrine disruption
	Mining	Direct toxicity
	Biomass burning	
Polycyclic Aromatic Hydrocarbons	Automotive	Formation of DNA adducts
	Industry	Impaired DNA replication and transcription
	Petrol extraction	
	Biomass burning	
Pesticides	Agriculture	Direct toxicity
		Endocrine disruption
		Formation of DNA adducts
Dioxins	Waste incineration	Direct toxicity
	Industry	Mutations
		Endocrine disruption
Polychlorinated Biphenyls	Industry	Direct toxicity
		Mutations
		Endocrine disruption
Solvents	Plastic Industry	Direct toxicity
		Oxidative stress
		Mutations
		Endocrine disruption
Trihalomethanes, Chlorophenols	Water disinfection	Mutations
		Endocrine disruption

3. Alterations of the sex ratio

The proportion of male to female live births (sex ratio) has been proposed to be an indicator of environmental contamination, probably due to the effects of endocrine disrupting substances. An eloquent example of such a situation is provided after the contamination by 2,3,7,8-tetrachlorodibenzo-p-dioxin near the region of Seveso, Italy (Mocarelli *et al*. 1996). Subsequently, the same group continued the study (Mocarelli *et al.,* 2000) to determine the levels of contamination causing the changes in sex ratio that were observed, and whether the parents' sex and/or age at exposure

were any significant effect modifiers. The results indicated that more female births took place after the accident, and that this effect was related to increasing TCDD concentrations in the serum samples from the fathers (20 ng per kg bodyweight; Mocarelli *et al.,* 2000). Also fathers exposed when they were younger than 19 years of age, sired significantly more girls than boys (Mocarelli *et al.,* 2000). Exposures to other agents, such as pesticides, inorganic borates and carbon, have also been associated with low sex ratios (James *et al.,* 1994; Milham *et al.,* 1993; Ryan *et al.,* 2002; Whorton *et al.,* 1977, 1994). Altered sex ratio was also observed in non-accidental conditions of environmental pollution. For instance, maternal exposure to polychlorinated biphenyls from contaminated fish from the Great Lakes decreases the sex ratio of offspring in a dose response-related way (Weisskopf *et al.,* 2003).

The mechanisms responsible for the alterations in sex ratio induced by environmental contamination are not yet clarified. James *et al.* (1990) proposed that exposure to environmental estrogens induces high gonadotropin and low testosterone levels, a situation that may promote injury and causes skewed the Y-bearing gametes before conception. The mechanisms responsible for such alteration are not hitherto clarified. In a recent review, Jongbloet *et al.* (2002) proposed that alterations of sex ratio caused by dioxin are due to its antiandrogenic properties, which alter the sperm-transit time, causing a delay of fertilization of the oocyte, in combination with deleterious sublethal X-linked genes, which are not compensated for in males by a second X-chromosome with normal genes. Interestingly, exposure to organochlorine pollutants was associated with increased proportion of human sperm Y:X chromosome ratio, suggesting that, although in higher proportion, the capacity of fecundation of Y chromosomes may be altered in consequence to toxic exposure (Tiido *et al.,* 2005).

Whether exposure to low levels of chemical substances during prolonged periods modifies sex ratio, as occurs in large urban centers, is a point of dispute. Ambient levels of air pollution were shown to increase sperm aneuploidy, mainly YYX disomy, a situation that favors abortion of male fetuses (Sram *et al.,* 2005). In a recent study, mice exposed to ambient levels of air pollution presented a reduced proportion of male births in comparison with controls (Mohallen *et al.,* 2005).

4. Increased rate of abortions and congenital anomalies

Reproductive adverse effects may be detected by assessing the rate of abortions and congenital malformations experienced by the exposed population. Contaminants of water, air and food were shown to be associated with abortions and malformations.

The effects of air pollution in determining the evolution and progression of pregnancy are of great importance, since gaseous emissions influence a large area and, consequently, thus affecting large populations. In such scenario, even modest risks may cause significant deleterious effects in terms of absolute numbers of affected individuals. There is evidence to support the role of air pollution in causing malformations and abortions provided by studies conducted in different areas of the world. The complex mixture present in the emissions of the petrochemical industry was associated with 2.9 times higher risk of spontaneous abortion in China, mostly due to exposure to benzene, gasoline and hydrogen sulphide (Xu *et al.,* 1998). Fetuses of mothers living in the area of influence of gaseous emissions of landfills in Cumbria, UK, presented a modest (odds ratio = 1.14, 95% confidence interval = 1.03 to 1.25) increase in the rate of congenital anomalies of the nervous system (Dummer *et al.,* 2003). Urban levels of air pollution were also associated with increased rate of abortions in São Paulo, Brazil (Pereira *et al.,* 1998). Coherently with the aforementioned findings, exposure to emissions derived from coal burning in Poland induces a greater number of DNA adducts with polycyclic aromatic hydrocarbons, the intensity of such adducts being inversely related to head circumference (Perera *et al.,* 1998). DNA adducts were also detected in greater number in placentas of fetuses in areas with high levels of air pollution in the Czech Republic (Topinka *et al.,* 1997), indicating that inhaled hydrocarbons with potential to interfere with DNA replication and transcription interact with fetal tissues.

Inhaled toxic metals also cross human placentas and reach the fetus. In Poland, lead and cadmium, two characteristic metals of urban air pollution, were demonstrated in high levels in placenta and fetal blood, in areas with heavy pollution (Baranowska, 1995). It is important to notice that levels as low as 10 µg/dl of lead in maternal blood are associated with increased risk of spontaneous abortion (Bellinger, 2005). As already mentioned, recent studies reported that mice exposed to ambient levels of air pollution in industrial locations exhibited increased frequency of heritable DNA mutations (Sommers *et al.,* 2002) and that reduction in pollution levels reverse the transmittance of such mutations to siblings (Sommers, 2004). Conceivably, these genetic abnormalities could increase the probability of disturbances in embryonic development, thus favoring abortions or congenital malformations.

Water contaminants also represent a risk in terms of malformations and abortions. The more evident situation is represented when the source of contamination is the spill of an industrial plant. Pregnancies of women living in areas receiving the effluents of oil companies operating in the Amazon basin have a 2.5 fold greater risk resulting in spontaneous abortion (San Sebastian *et al.,* 2002). The contamination of public water by long-lasting industrial solvents is consistently associated with increased rate of abortions and malformations (Deane *et al.,* 1989). In this case, the situation is particularly problematic, since the contamination is not always evident: the plume of leaking industrial residues may reach the reservoirs of subterraneous water used by a large number of people. Also, the detection of contamination may occur after a long period of exposure.

Pesticides are unequivocally associated with increased risk of malformations and increased rate of abortions (Cavieres, 2004; Salazar-Garcia *et al.,* 2004). Pre-conception exposure of mothers to pesticides is associated with early abortion, whereas post-conception exposures tend to be related to late fetal losses (Arbuckle *et al.,* 2001). Food contamination by pesticides is also associated with impaired pregnancy evolution, as regrettably demonstrated by the accidental exposure of women to contaminated grain containing high levels of hexachlorobenzene in Turkey (Jarrell *et al.,* 1998). However, the risk associated with lower exposure levels found in the general population remains uncertain, and epidemiological studies addressing the possible reproductive consequences of pesticide contamination in food products are badly needed

A point that has evoked a significant degree of concern is the potential adverse reproductive effects of water disinfection products, due to the large population exposed to such contamination. The most used procedure to disinfect water for public use is chlorination. Chlorine may react with organic substances, forming several halogenated compounds, such as chloroform, trihalomethanes, chlorophenols and brominated trihalomethanes (Nieuwenhuijsen *et al.,* 2000). There is significant toxicological evidence to support the concept that such compounds may affect human fertility and embryonic development (Nieuwenhuijsen *et al.,* 2000), but the number of human epidemiological studies in this area is still small. Increased risk for abortions was detected by some authors in the population exposed to trihalomethanes (Waller *et al.,* 1998). Several studies demonstrated significant associations between exposure to chlorinated products in public water and congenital defects, mostly urinary and neural malformations (Aschengrau *et al.,* 1993; Bove *et al.,* 1995; Dodds *et al.,* 1999; Hwang *et al.,* 2002; Klotz and Pyrch, 1999; Magnus *et al.,* 1999; Wrensch *et al.,* 1992). Although there are some limitations in determining precisely the exposure level and sources of contamination

by water disinfection products (inhalation and dermal exposures), the evidence of adverse effects of such substances are strong enough to deserve attention, as well as and the implementation of control measures to protect public health.

5. Preterm delivery and low birth weight

An important point in environmental toxicology relates to the effects of ambient levels of air pollution on fetal development. To this end, birth weight, and time of gestation, is commonly used indicators. The large number of people exposed to air pollution points out that, although the effects of air pollution may be small, the risk attributed to air pollution can be high. Moreover, the highest levels of air pollution are recorded in the large urban areas of developing countries, and in areas that use biomass burning for cooking purposes. In these ecosystems also other detrimental factors to pregnancy – economic deprivation, multiple gestations, famine – are present, and may act in synergy with the environmental contamination. Large medical registries, combined with continuous measurements of ambient air pollutants, showed that air pollution has adverse effects on fetal growth and gestational age. Increases in the risk of low birth weight (birth weight <2500 gr), very low birth weight (birth weight <1500 gr), intrauterine growth restriction (birth weight below the 10th percentile of birth weight for gestational age and sex), and preterm delivery (delivery <37 weeks of gestation). Recent reviews (Maisonet, 2004; Sram, 2005) allow concluding that these epidemiological associations are causal, and not fallacious.

While epidemiological evidence now may relate air pollution and impairment of fetal growth in a causal way, there is still insufficient information on the mechanisms responsible to establish a correlation between the impact of fetal exposure to air pollution and the risk to develop disease during later stages of life. In other words, do children born in areas with high levels of air pollution, showing impairment of growth during gestation, also have an increased risk to develop diseases in their adulthood? Does impairment of the development of an organ result in health problem later? These questions are key points to characterize the real impact of ambient levels of air pollution on fetal development.

6. Conclusions

Despite the uncertainties in assessing exposure and the magnitude of risk, the evidence supports that environmental contamination due to toxic chemical species impacts adversely on human reproduction. Several reproductive end-points are

indicative of such harm, like decreased production and quality of male and female gametes, as well as increased rate of genetic abnormalities of oocytes and spematozoa, which may, ultimately, lead to increased abortion and congenital malformations. Another important indicator of reproductive impairment is the alterations in the sex ratio (proportion of males in respect to total born alive) that is observed in some populations exposed to environmental toxicants. Table 6 summarizes the reproductive alterations associated to environmental contamination.

Table 6. Adverse reproductive end-points associated to environmental contamination

Decreased ability of fertilization	Low birth weight
Decreased production of gametes	Increased rate of abortions
Genetic abnormalities of gametes	Hormonal disruption
Prematurity	

A long way is open to future research aiming to elucidate the mechanisms and relevance of the observed impacts of environmental contamination on fertility. In some selected (as well as tragic) situations, the relevance of environmental impact is obvious, due to its severity and magnitude (such as occurred in the contamination of people in Seveso, Italy, by dioxins). This is not true in most cases: for instance, do the decreases in sperm counts (if proven true) represent a real problem for human fertility? What is the magnitude of the impact of diffuse contaminations, such as air pollution or water disinfection byproducts, on reproductive function? How much do these intra-uterine, pre-conception exposures predispose to future disease? In addition to these questions, there is an urgent necessity to study the mechanisms responsible for fertility impairment caused by different classes of environmental toxic substances. In this category, the problem of endocrine disrupting substances is one of the most challenging ones. Endocrine disruption is the resulting action of agents with quite different molecular structures, like cadmium and pesticides, indicating that the magnitude of endocrine disruption cannot be adequately predicted *a priori* by examining the type of molecule and molecular configuration. These difficulties pose an intriguing and challenging scenario for environmental toxicologists, and for those interested in providing a better world for human kind in the future.

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SECTION 2:

REPRODUCTIVE HEALTH EFFECTS

MALE REPRODUCTIVE STATUS AND ITS RELATIONSHIP WITH MAN-MADE, HORMONE-DISRUPTING SUBSTANCES: *STUDIES IN FLANDERS, BELGIUM*

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Summary

Sperm quality seems to have declined over time in certain regions in the world. This regional trend coincides with the progressive increase in the incidence of male genital tract anomalies including testicular maldescent, hypospadias and testicular cancer. The association of these pathologies has been called the 'testicular dysgenesis syndrome' (TDS). The possible implications of decreased male fertility cannot be underestimated. Couples attending fertility clinics have a higher chance of multiple pregnancies, preterm delivery and consequently also of low birth weight, which is a known risk factor for a number of pathological conditions at adulthood. Many studies suggest TDS to be caused by man-made endocrine disrupters, mostly chemicals initiating estrogen actions (xenoestrogens) or inhibiting the effects of endogenous androgens (anti-androgens). Thousands of man-made chemicals have been released into the environment in vast quantities since the chemical industry began to boom in the 1950´s. Humans and wildlife are exposed to these chemicals through their nutrition, the air, the water and, more importantly, through the placenta during foetal development. In order to reliably estimate the reproductive health risks posed by these chemicals, scientific programmes should pursue the identification of the routes via which humans and wildlife are exposed to these xenobiotics using biomarkers of internal and external exposure. These are new and important tools, complementary to the traditional chemical analytical techniques as they reflect the integrated response of an individual or test organism to multiple compounds, accounting for possible additive, antagonistic or synergistic effects.

P. Nicolopoulou-Stamati et al., (eds.), Reproductive Health and the Environment, 75–94.

1. Introduction

Male sexual differentiation and development as well as male fertility and sexuality are under tight endocrine regulation by the hypothalamo-pituitary-testicular (HPT) axis. Any factor disturbing this axis may result in male gonadal dysfunction. A temporal decline of male fertility in humans has been documented in many studies (Carlsen *et al.,* 1992; Van Waeleghem *et al.,* 1996; Irvine *et al.,* 1996; Swan *et al.,* 2000). These changes in male fertility are paralleled by an increased incidence of "endocrine dependant" pathologies in the male including hypospadias, cryptorchidism and testicular cancer, collectively termed the "testicular dysgenesis syndrome" (Skakkebaek *et al.,* 2001). A recent study suggests that Leydig cell dysfunction may also be a component of this syndrome (Andersson *et al.,* 2004). Jensen (2002) argued that poor semen quality may contribute to the recent decline in fertility rates observed in the industrialized world.

Subfertility, generally defined as a failure to conceive after one year of unprotected regular sexual intercourse, is a common problem affecting 15-20 per cent of all couples in Europe (Tuntiseranee *et al.,* 1998; Karmaus and Juul*,* 1999; Joffe*,* 2000; Taylor, 2003). The likelihood of spontaneous conception is affected by numerous factors including male and female physiological conditions, genetic defects, sociodemographic evolutions and probably environmental factors. In the last decades subfertile couples tend to appeal more often to assist reproductive techniques, frequently resulting in multiple pregnancies (Reynolds *et al.,* 2003; Sutcliffe, 2002). Multiple pregnancies are a risk factor for preterm delivery, and consequently also for low birth weight (Barad and Witt*,* 2000), increasing the odds for the offspring of developing diabetes and heart disease later in life (Eriksson *et al.,* 2004; Rich-Edwards *et al.,* 1999). Moreover, there are strong indications that even in singleton pregnancies, the odds for several adverse perinatal outcomes are significantly higher in assisted compared to spontaneous conceptions (Jackson *et al.,* 2004; Helmerhorst *et al.,* 2004). Finally, several recent publications indicate a higher risk of congenital abnormalities, of impaired neurological development, and –possibly- of certain cancers for children born after IVF compared to others (Hansen *et al.,* 2002; Stromberg *et al.,* 2002; Sorensen *et al.,* 1997; Moll *et al.,* 2003).

Like most disorders, infertility is the endpoint of a sometimes synergistic action of diverse pathogenic factors. Broadly, for male infertility, these can be categorized into 4 groups a) chromosomal and genetic defects, (e.g. Klinefelter syndrome and Y chrosome microdeletions) b) life style (e.g. smoking, alcohol abuse, drugs) c) classical medical disorders (e.g. varicocele, accessory gland infections) d) The environment in which one lives or works (Quintana-Murci and Fellous, 2001;

Sharpe and Franks, 2002; Matzuk and Lamb, 2002). Over the years, we have engaged in a number of studies related to the environmental impact on male reproductive status. This report does not aim to be exhaustive and fully comprehensive but merely describes, and puts into perspective, the results of our endeavours to investigate 1) the impact of specific occupational exposures on sperm quality, 2) time trends and 3) regional differences in sperm quality in Flanders and finally 4) the presence and biomarker based identification of estrogenic substances in the Flemish environment.

2. Observations on male reproductive status

Spermatogenesis is a complex cyclic process of mitosis, meiosis and morphological differentiation by which germinal cells mature into elongated spermatozoa (Zhang *et al.,* 2003). Its delicate spatio-temporal regulation is the result of a cellular interplay between Sertoli, Leydig and germ cells under the direct control of the pituitary hormones FSH and LH. The latter hormone stimulates the Leydig cell production of testosterone (T) resulting in the high interstitial and seminal tubule T levels necessary for a successful spermatogenesis. Semen analysis has been part of the standard diagnostic routine for infertile couples ever since low sperm count was linked to male infertility. However, in fertile populations, little is known about the association between semen parameters and time to pregnancy. Recent epidemiological studies have highlighted the importance of sperm morphology on the couple's ability to conceive (Bonde *et al.,* 1998; Guzick *et al.,* 2001; Slama *et al.,* 2002). It has also been shown that sperm morphology is highly susceptible to a number of environmental and life style factors (Auger *et al.,* 2001).

In 1984, our laboratory performed a study in collaboration with the Department of Public Health of our University, to investigate the possible effects of carbon disulfide (CS_2) exposure on male fertility and semen quality. Forty-three out of 129 eligible viscose rayon workers and 35 out of 79 workers who were not exposed to any toxic agent in the working environment provided a semen sample that was investigated according to the World Health Organization standard method (Vanhoorne *et al.,* 1994). Eighty one percent of the CS_2 exposed men presented abnormal sperm quality. Unexpectedly, as many as 69 per cent of the control population also had poor semen quality, in particular an excessive number of spermatozoa with abnormal morphology. Concordingly, researchers of the University of Copenhagen reported a high frequency of sub-optimal semen quality in an unselected population of young men (Andersen *et al.,* 2000).

The question that arises is whether these alarming cross-sectional observations represent the stable male reproductive status of a country, or whether sperm quality measures have decreased with time in that population. A meta-analysis of a large number of published papers on semen characteristics of healthy men without a history of infertility, revealed a reduction to half of average sperm concentration (i.e. the number of spermatozoa per ml of ejaculate) over a period of 5 decades (Carlsen *et al.,* 1992) but this decrease has been questioned by others (Sherins, 1995). In Flanders, we have documented a dramatic decrease of sperm quality measures over an observation period of 19 years (Van Waeleghem *et al.,* 1996). Figure 3 extends these data and shows retrospectively analysed sperm characteristics of 456 consecutive healthy young men, aged 20-40 years, who presented themselves as candidate sperm donors between 1976 and 1998. Average sperm concentration presented only a minor decrease, which was compensated by a proportional increase of the ejaculate volume (data not shown). However the functional characteristics of spermatozoa, namely rapid linear sperm motility and sperm morphology, deteriorated to such an extent (Figure 3) that the proportion of allegedly normal men with sub-optimal sperm quality (below the WHO's lower limit of normality) increased from 5 to 45 per cent, and the percentage of men with frankly infertile semen (below the 5th percentile of sperm characteristics of subfertile men) increased five fold from 1.6 to 9 per cent (Figure 4**)** (Comhaire *et al.,* 1996). The fact that the correlation between sperm motility and sperm morphology was identical in the early as compared to the more recent samples suggests that both characteristics deteriorated in parallel, rather than changes in the technique of semen analysis being responsible for the observed decreases. The observed prevalence of approximately 9 per cent male infertility is in agreement with the observed 15-20 per cent prevalence of couple infertility, since a "male factor" is detected in half of these couples (Adamson and Baker, 2003). Furthermore, the average percentage of spermatozoa with normal morphology observed in the recent group of the candidate sperm donors is equal to that recorded among "normal" men investigated in population studies (Figure 5). Our findings in healthy candidate sperm donors are reinforced by studies performed in Paris (Auger *et al.,* 1995), Athens (Adamopoulos *et al.,* 1996), Scotland (Irvine *et al.,* 1996), and Denmark (Gyllenborg *et al.*, 1999) in similar cohorts of sperm donors or men attending infertility clinics. Furthermore, a recent publication has proven severe deterioration of testicular histology over a time period of 10 years (Pajarinen *et al.,* 1997). The conclusion that can be drawn from the epidemiological studies that have been published following the Carlsen article is that there seems to be spatial differences in the trend towards sperm deterioration (Swan *et al.,* 2000) as is also illustrated by the comparison between Paris and Toulouse (Auger *et al.,* 1995 vs Bujan *et al.,* 1996).

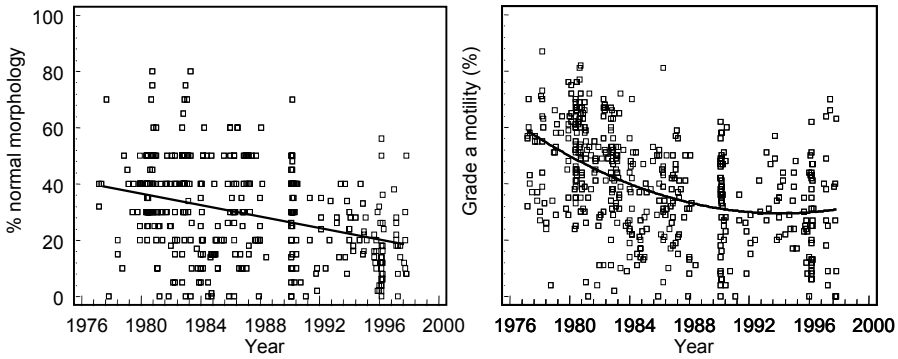

Figure 3. Percent of spermatozoa with rapid linear progressive motility (grade a) in healthy candidate sperm donors over an observation period of 20 years. The decrease is statistically highly significant, and clinically relevant (Van Waeleghem *et al.,* 1996).

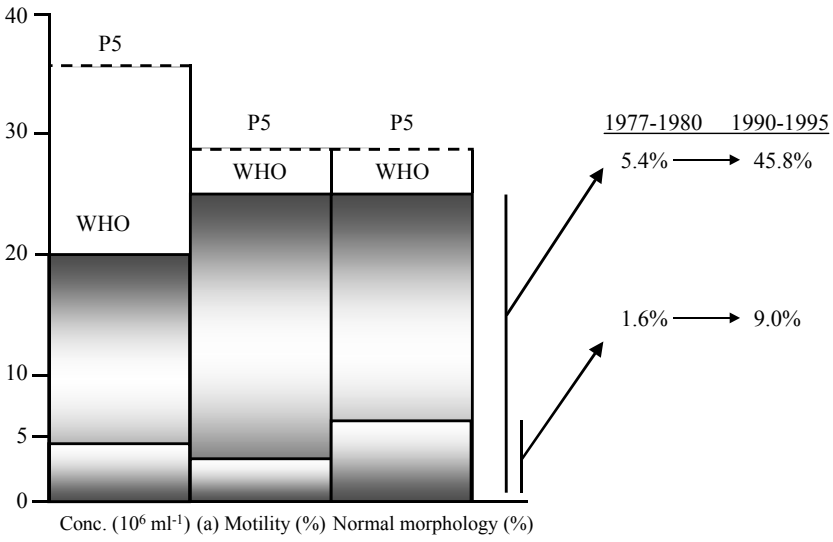

Figure 4. Figure representing the different cut-off points of conventional sperm characteristics of fertile and subfertile men. P5= 5th percentile of concentration, percentage rapid progressive (grade a) motility, and normal morphology of fertile men. WHO: lower limit of normality quoted in the WHO laboratory manual (1987). *: 5th percentile of subfertile men who ultimately attained conception (Comhaire *et al.,* 1996).

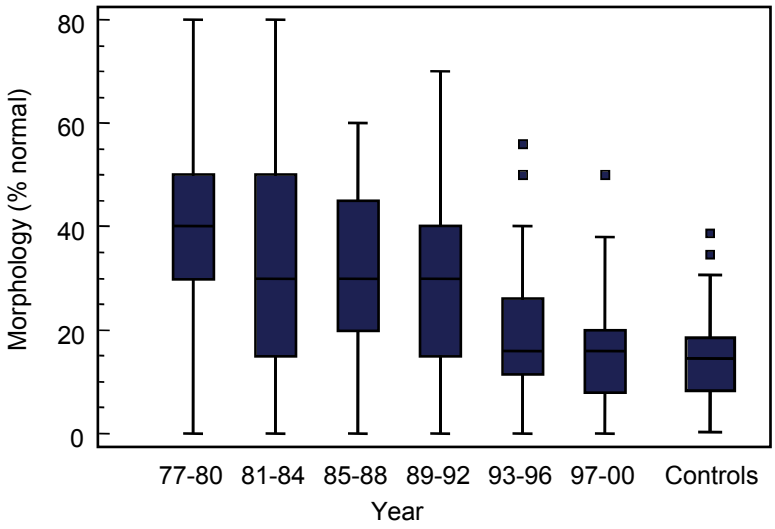

Figure 5. Sperm morphology (% normal forms) in candidate sperm donors over a time period of 2 decades, and in population studies. The data are represented in box and whisker plots (unpublished data).

Most studies on sperm quality are city based. In a large concerted European study it was shown that sperm parameters differed between four European cities (Jørgensen *et al.,* 2001). Little, however, is known regarding the differences between sperm quality in a rural vs an urban environment (Swan *et al.,* 2003a). In the framework of a large Environment and Health study, in which also adolescents and older women were investigated (Staessen *et al.,* 2001; van Larebeke *et al.,* 2004), we analyzed semen samples and determined serum sex hormone levels in healthy non-smoking men aged 21-40: 50 from two industrial suburbs of the city of Antwerp and 51 from Peer, a predominantly rural municipality 70 km east of Antwerp (Dhooge *et al.,* submitted). Persons with known occupational exposures, persons working in a region with characteristics clearly different from those of the area of residence, and people commuting over long distances were excluded from the study. In the rural compared to the urban area, participants had a lower sperm concentration (by 34%, $p = 0.06$), total sperm count (by 41%, $p = 0.02$) and sperm morphology (by 32%, $p<0.001$). Concordingly, free testosterone was significantly lower (11%, $p = 0.03$) in this area, while for total testosterone (10%) and FSH (17%) the differences were non-significant (both $p = 0.09$). Our data do not suggest a testicular factor causing the observed impaired semen quality status in the population in Peer. An undefined testicular threat leading to impaired testosterone secretion logically would have resulted in increased compensatory LH levels, which was not seen in this study. The lower FSH levels in Peer than in Antwerp do however support the possibility of

partial spermatogenic arrest at the spermatid level related to alter pituitary function. Complementary measurements have indicated the rural population to be exposed to higher concentrations of the pesticide DDT than the men from the urban area. These observations sustain the hypothesis of environmental factors causing testicular malfunction.

We have engaged in additional studies to detect possible damaging effects of professional and/or environmental exposure on sperm quality, and testicular function in general. Semen analysis was performed in 68 men working in the lead-contaminated environment of a lead melting factory, and in 91 "unexposed" controls working at the Ghent University Hospital. Lead workers had significantly lower sperm concentration (35 vs. 51 million/mL) and higher serum inhibin B (259 vs. 177 pg/mL) as well as lead blood levels (30.9 vs. 3.4 mug/dL) compared with the hospital personnel (all $p < 0.05$). Serum FSH and E2 levels were similar in both groups. Overall, inhibin B levels correlated significantly positively with blood lead levels and sperm concentration while it was negatively correlated with serum FSH. The results of the present study suggest that the exposure of the cells of Sertoli to excessive amounts of lead results in inappropriate inhibin B overproduction that may be involved in the impairment of spermatogenesis (Mahmoud *et al.,* 2005).

3. Search for the causal factor(s)

The impressive regional deterioration of testicular histology and function is paralleled by a dramatic increase in the prevalence of testicular cancer. The incidence of this disease has increased three fold over the last 50 years in Denmark, and a similar phenomenon was observed in several other (northern) European countries and the US (Adami *et al.,* 1994; Mckiernan *et al.,* 1999). Also, in these populations rising trends in hypospadias and possibly cryptorchidism have been described (Paulozzi, 1999; Toppari *et al.,* 2001). It is argued that these disorders, which share many risk factors as well as being risk factors for each other, may have a common aetiology. Scientists have consequently searched for a unifying theory to explain the (regional) rise in prevalence of these male reproductive abnormalities. The fact that they have increased in many countries over a short period of time, rather seems to reflect changes in environmental factors or "life style" than genetic factors (Carlsen *et al.,* 1992; Jensen *et al.,* 1995; Skakkebaek *et al.,* 2001).

Although for many years there have been a number of reports associating exposure of wildlife to anthropogenic chemicals and reproductive failure and problems with the development of the young, it is only since 1991 that a common thread could link

these problems in wildlife. Many of the observed effects were synonymous with what would be expected from disruption of the body's hormones. Decreased hatching success, reproductive abnormalities, decreased fertility and behavioural abnormalities in fish, turtles, birds and/or mammals were not only recorded in species inhabiting heavily polluted areas such as the Canadian Great Lakes or Lake Apopka in Florida, but were also evident in wildlife in many other regions of the world (Wingspread, 1991; Colborn *et al.,* 1993; Cooper and Kavlock, 1997; Crisp *et al.,* 1998; Fry, 1995; Guillette *et al.,* 1994).

At the average age when women in modern countries conceive for the first time, being near 30 years, there has been an important accumulation of xenobiotic persistent substances in their body and some of them can pass freely through the placenta (Foster *et al.,* 2000). In 1993 Sharpe and Skakkebaek re-stated and expanded a previously published hypothesis on the origin of testicular cancer saying that an increased fetal exposure of the male to estrogenic compounds could lead to cryptorchidism, hypospadias, testicular cancer and possibly decreased sperm quality (Sharpe and Skakkebaek, 1993). An over-exposure to estrogenic compounds might result, through various mechanisms, in a sub-optimal fetal development and maturation of the cells of Sertoli, germ cells and Leydig cells, leading to a dysgenetic testis, impaired in its transabdominal descent and in its support of spermatogenesis and populated with persistent embryonic cells that may deteriorate into testicular cancer later in life. Indeed, abnormalities similar to the ones observed in men, can be produced experimentally in animals by exposing these to so called xenoestrogens. The prototype of the latter is diethylstilbestrol (DES), a synthetic molecule with estrogen-like effects in humans and animals, but with a chemical structure completely different from that of the natural 17-beta-estradiol. When DES is given in utero or neonatally, the male offspring in adulthood present a number of reproductive disorders including reproductive tract tumors, reduced testis weight, rete testes abnormalities, impaired spermatogenesis and mating behaviour in several mammalian species (Goyal *et al.,* 2003; Atanassova *et al.,* 1999; Khan *et al.,* 1998). In humans, the sons born from mothers who were treated with DES during a particular period of pregnancy, present abnormal genital development and a decreased sperm quality (Klip *et al.,* 2002; Gill *et al.,* 1977; Bibbo *et al.,* 1977).

It was realised that other man-made chemicals had a DES-like estrogenic effect, at least in *in vitro* experiments. Agricultural pesticides, industrial and/or household chemicals such as certain detergents, plasticizers and polychlorinated biphenyls (PCBs) or their metabolites have been described to interact with the estrogen receptor (Soto *et al.,* 1995; Crisp *et al.,* 1998; Miller *et al.,* 2001). Recent studies suggest that some heavy metals also behave like estrogens *in vitro* as well as *in vivo*

(Stoica *et al.,* 2000; Johnson *et al.,* 2003). Finally, some halogenated polyaromatic hydrocarbons are described to increase the bio-availability of endogenous estrogens in target tissues through the inhibition of the enzymes involved in estrogen inactivation (Kester *et al.,* 2000). Some of the above described DES effects could be reversed by testosteron co-administration (Rivas *et al.,* 2003). The latter interesting observation substantiates other plausible mechanisms leading to the testicular dysgenesis syndrome, namely suppression of androgen production, androgen receptor expression or the synthesis of Leydig cell insulin-like factor-3 (Sharpe, 2003). A growing number of xenobiotics, including some pesticides and phthalates seem to interact with the androgen receptor (Kelce *et al.,* 1995; Sohoni and Sumpter, 1998; Andersen *et al.,* 2002). Moreover a number of compounds have been described to exert anti-androgenic effects via several distinct mechanisms when administered pre- or neonatally in laboratory animals (Wolf *et al.,* 1999). TDS could be elicited experimentally in rats exposed *in utero* to dibutylphthalate (Fisher *et al.,* 2003). Endocrine disrupters have consequently been defined as *'exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour'* (Kavlock *et al.,* 1996). The present information however, is insufficient to link with certainty the above described alarming reproductive effects in humans to exposure to certain man-made chemicals. This lack of information is caused by the complexity of the endocrine system on the one hand, and by the low levels at which chemical substances can interact with the endocrine system on the other hand. However, recent epidemiological studies have revealed associations between sperm parameters and pesticides (Swan *et al.,* 2003b), phthalates (Duty *et al.,* 2003), and polychlorinated biphenyls (Hauser *et al.,* 2003; Hsu *et al.,* 2003).

4. Effects on postnatal human development

Spermatogenesis, as well as testicular function in general, is highly vulnerable. Any intrinsic physical disease, either involving the genital organs or the general physical condition, may cause sperm deterioration. Postnatal exposure to xenoestrogens can suppress the hypothalamo-pituitary secretion of gonadotropins directly and indirectly through the increased production of inhibin B by the cells of Sertoli. The data from our population studies show that men working in the lead contaminated metal factory present a higher inhibin B concentration in blood, in spite of a lower sperm concentration, indicating a direct effect of this heavy metal on the cells of Sertoli. The increased inhibin B suppresses FSH, which should normally have been stimulated because of the lower sperm concentration.

Treating infertile men with the antiestrogen tamoxifen (possibly in association with an androgen; Adamopoulos *et al.,* 2003) can reverse the suppressive effects caused by the xenoestrogens, and restore sperm quality and fertility in a proportion of men with so called idiopathic oligozoospermia. This treatment may turn out to be less effective in cases where the prenatal development of the testicular "anlage" has been dysgenetic. Flanders has a tradition of intensive agriculture and use of pesticides (Peeters *et al.,* 2004), and a dense network of waste incinerators as well as chemical and textile factories, some of which have produced and discharged large amounts of hormone disrupters. The data on the internal exposure of the populations of Peer and Antwerp (*vide supra*) have indicated increased concentrations of polyaromatic hydrocarbons, compounds with dioxin-like biological activity and – most of all – chlorinated pesticides (including the forbidden DDT) in the older inhabitants of the rural area, many of whom consume vegetables from their own gardens (van Larebeke *et al.,* 2004; Koppen *et al.,* 2002). Some of these agents can exert an oestrogen – like effect that stimulates inhibin B secretion by the cells of Sertoli (Depuydt *et al.,* 1999), which in its turn suppresses the FSH response at the hypothalamo-pituitary level.

Phenomenal amounts of xenobiotics are being manufactured, and their presence in the environment is universal. Some of these substances are very persistent and have accumulated over long periods of time, both in the environment, the food chain and consequently in the fat tissue of the human and animal body. Xenoestrogens may exert an additive or synergistic effect and they can enhance the effectiveness of the endogenous 17-beta-estradiol by interfering with its metabolism (Rajapakse *et al.,* 2002; Kester *et al.,* 2000; Arnold *et al.,* 1996). In addition, the capacity to deregulate biological processes may be reinforced by the simultaneous exposure to xenoestrogens and anti-androgens (Rivas *et al.,* 2002). Thus, there are good reasons to believe that hormone disruption can result from agent concentrations in water and food that were considered safe in the past.

5. Searching for the sources of xenoestrogens

Several bioassays have recently been developed to identify anthropogenic and natural compounds that are able to bind to steroid receptors and elicit a hormone - specific response. These low-cost, high throughput screening assays have also found their application in the toxicity evaluation of environmental samples. However, the multitude and the structural diversity of endocrine disrupters and the possible antagonistic or synergistic interactions of these compounds in the assay make identification of the responsible chemicals by chemical analytical means difficult.

Bioassay-directed fractionation procedures followed by high pressure liquid chromatography (HPLC) and/or gas chromatography coupled with mass spectrometry (MS) are a more successful means of associating sample toxicity with compound identification.

Over the past few years, our laboratory has implemented and validated existing, and developed new *in vitro* assays for estrogenic activity. In 2003, we finalized a three years co-operation project between our department, the Flemish Institute for Technological Research (VITO) and the Free University of Brussels. The objectives of this study were to 1) make an inventory of the emissions and environmental distribution of substances with hormone disrupting potential in Flanders, 2) screen Flemish waters using *in vitro* and *in vivo* assays for estrogenic activity 3) identify the compounds responsible for the toxicological response and 4) evaluate the risk of human exposure to a number of these hormone disrupters (Witters *et al.,* 2003). As part of this study we have investigated surface water samples from 32 different points of the Upper Schelde River at different points in time. After suitable extraction procedures, the samples were tested in two different *in vitro* systems for estrogen activity based on either a genetically modified breast cancer cell line (MVLN) or genetically manipulated yeast cells (YES) (Demirpence *et al.,* 1993; Routledge and Sumpter, 1996). A limited number of samples were also tested in an *in vivo* fish test measuring estrogen induced vitellogenin production in Zebra fish. In addition, samples were assessed in parallel to the MVLN and YES using a cell-free, competitive human estrogen receptor binding assay (ERBA) developed in our laboratory. A sophisticated liquid chromatographic tandem mass spectrometric (LC-MS/MS) method was developed (Benijts *et al.,* 2002) and implemented to identify the agents with estrogenic activity detected in the water samples. In this study it was found that although the concentrations of estrogenic compounds, measured using the YES and expressed as ng estradiol equivalent (ng E2 eq.) in the effluents of sewage treatment works were equal or lower (0.87-14.4 ng E2 eq.) than the ones measured in Sweden (0.1-15 ng E2 eq.; Svenson *et al.,* 2003), the United States (<1-15 ng E2 eq.; Huggett *et al.,* 2003 or 21-147 ng E2 eq.; Tilton *et al.,* 2002), Germany (11.8-20.2 ng E2 eq.; Pawlowski *et al.,* 2003) or the Netherlands (3.5-7.9 ng E2 eq.; Vethaak *et al.,* 2002), the concentrations in surface waters (0.55-69.7 ng E2 eq.) were in general 10 to 50 times higher than the values reported in the above cited-studies. The highest value of almost 70 ng E2 eq. was 70 and 350 times higher than the concentrations measured in Germany and the Netherlands respectively, but was in close agreement with previously reported values from the same river (Tanghe *et al.,* 1999). We also detected very high estrogen activity in industrial effluents (165 ng E2 eq.) using the ERBA, but not the YES nor the MVLN, possibly related to toxicity problems in the latter two cell- based systems. Results in all three assays

were highly comparable for the non-industrial samples. Similar toxic responses were noted in the *in vivo* assay, whereby fish were exposed to surface water samples from the same location as the ones that were investigated in the *in vitro* assays. We subsequently developed a bioassay directed fractionation procedure, in which estrogenic environmental samples were fractionated on a reversed phase (octadecyl) HPLC column, and the estrogenic activity was measured in the resulting 30 fractions. In addition the samples were analyzed by LC-MS/MS for 35 endocrine disrupters, including the natural estrogens 17β-estradiol and estrone and the synthetic compounds 17α-ethinylestradiol, a number of parabens and alkylphenols. We could statistically link the estrogenic activity in a number of HPLC fractions to the LC-MS/MS determined concentrations of parabens and alkylphenols. These results are surprising in view of the fact that, to the best of our knowledge, only in sediment samples has it been possible to show a relationship between the concentration of alkylphenols and the estrogenic activity in the samples. (Fenet *et al.,* 2003). No comparable data are available on parabens. This highlights the importance of screening environmental samples using bioassays, as this could reveal toxic compounds that were previously unknown to exist in these samples.

Overall, this project showed that using assays for estrogenic activity that are based on cell culture can give reliable results in environmental samples that display little estrogenic activity, and are devoid of toxic effects. For toxic samples, the use of non-cellular receptor based test systems is advisable.

6. Conclusions

There is firm evidence supporting the concept of environmental causes for the dramatic increase in male infertility. The medical techniques of assisted reproduction used to overcome this epidemic of infertility, are burdened with serious hazards that may hypothecate the future health and quality of life of both the mother and of the children. Since the level of contamination of our environment by hormone disrupting agents is so overwhelming, and many of these substances are highly persistent, methods must urgently be developed and implemented to reduce the (human) exposure to, and uptake of hormone disrupters.

Acknowledgement

This work has benefited from financial support given by Ghent University. Grants were also received from the Asclepios European project, Life98 European project and the Flemish Government. Willem Dhooge is supported by a fund from the

Support Group Environment and Health, financed by the Flemish Government (Department of Science, Department of Public Health and Department of Environment, Brussels, Belgium). The contribution and assistance is acknowledged of N. De Clercq, K. Van Waeleghem, S. Stuyvaert and F. Schoonjans, and the Department of Public Health, Ghent University Hospital.

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POTENTIAL IMPACT OF THE ENVIRONMENT ON THE MALE REPRODUCTIVE FUNCTION: *THE EXAMPLE OF CRYPTORCHIDISM*

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Summary

Cryptorchidism is the most frequent genital anomaly in male newborns (> 1 per cent). Being small for gestational age seems to be the main risk factor for cryptorchidism. Recent warnings coming from industrialized countries have placed emphasis upon an increasing incidence over the last decades in male reproductive abnormalities (cryptorchidism, hypospadias, and testis cancer) and, on the contrary, a reduction in sperm counts. Testicular cancer, cryptorchidism, hypospadias and poor spermatogenesis are risk factors for each other and it has been hypothesized that they are all signs of a developmental disturbance called testicular dysgenesis syndrome.

Although genetic abnormalities can cause cryptorchidism, in the majority of cases, the reasons remain unclear. Eco-epidemiological studies suggest that environmental factors contribute to the problem. The main hypothesis is that cryptorchidism (like other disorders) is consecutive to exposure of the developing foetus to "endocrine disrupters". These chemicals with endocrine activity have become a prime importance topic of investigation.

The purpose of this review was to present and analyse the level of evidence of the relation between endocrine disrupting chemicals and cryptorchidism.

P. Nicolopoulou-Stamati et al., (eds.), Reproductive Health and the Environment, 95–118.

In animals, the level of evidence for an impact of endocrine disrupters on cryptorchidism is high, and biological pathways by which chemicals may disrupt endocrine balance are better identified: various environmental factors hav demonstrated their ability to mimic, antagonise or interfere with androgens and estrogens, or to disrupt the physiological estrogens/androgens balance.

Even if evidence in humans remains limited, recent epidemiological studies improve the level of evidence for an exposure-outcome relation between endocrine disrupters and the occurrence of cryptorchidism.

Further researches focusing on endocrine modulation are desirable, in order to ensure full validation of effects of endocrine disrupters.

1. Introduction

Recent warnings coming from several industrialized countries have emphasized upon an increasing incidence over the last three decades in male reproductive abnormalities (cryptorchidism, hypospadias, testis cancer), and on the contrary a reduction in sperm counts (Carlsen *et al.,* 1992; Auger *et al.,* 1995; Van Waeleghem *et al.,* 1996; Irvine *et al.,* 1996; Adami *et al.,* 1994; Bergstrom *et al.,* 1996). One main hypothesis is that these disorders are consecutive to exposure of the developing fetus to "endocrine disrupters" (Sharpe and Skakkebaek, 1993; Whorton *et al.,* 1979; Ratcliffe *et al.,* 1987; de Cock *et al.,* 1994; Thonneau *et al.,* 1999; Cheek and McLachlan, 1998).

According to the definition of the IPCS Steering Group that met at the joint IPCS/OECD Scoping Meeting on Endocrine Disrupters, 16-18 March 1998 in Washington, DC, an endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or populations.

A potential endocrine disrupter is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or populations (Campbell and Hutchinson, 1998).

Alteration of endocrine function caused by an endocrine disrupter may be through interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior. Many suspected endocrine disrupters are industrial chemicals, consumer chemicals and chemicals in the

environment that can mimic, enhance (an agonist) or inhibit (an antagonist) the action of hormones.

This has led stakeholders to consider the topic of endocrine disruption as of sufficient concern to justify action.

Among them can be mentioned the European Commission, the European Parliament, the US Environmental Protection Agency, OECD, the IPCS, the WHO, the Commission of the OSPAR Convention, the European Environment Agency, NGOs and the chemical industry.

Exposure during intrauterine life represents a very likely window of susceptibility to endocrine disrupters, because sensitivity to any endocrine disturbance is increased during prenatal period. Therefore, cryptorchidism, which is undoubtedly a birth defect of prenatal origin, represents an ideal model.

The aim of this article is to summarize the current knowledge about mechanisms of cryptorchidism genesis, to review the endocrine disrupter hypothesis and to evaluate the level of evidence of a potential relationship between deleterious environmental (or occupational) conditions, and the occurrence of cryptorchidism.

2. Cryptorchidism: epidemiology and biology

2.1. Definition of cryptorchidism

According to Scorer (1964) “the descent of the testis is the descent of an organ from the abdominal cavity to the bottom of a fully developed and fully relaxed scrotum”.

Cryptorchidism (which in Greek means “hidden testis”) is characterized by the failure of one or both testes to descend into the scrotum, despite being on the right way.

Cryptorchidism is the most frequent male sexual anomaly. It is the main risk factor for testis cancer, which is the first cancer in young men (Huyghe *et al.,* 2003), and it is also a major risk factor for male infertility (Scorer, 1964; Chilvers *et al.,* 1984). Spontaneous descent of testes happens in 70 per cent of cases during the first year of life. So, an overestimation of the number of cryptorchid children is made possible in the case of early evaluation of incidence of cryptorchidism. Another confounding, as pointed out by Toppari *et al.* (1996). Some authors consider as “normal” a location

of the testis in a high scrotal position, whereas others include retractile pushed into this position) within cases of cryptorchidism.

Moreover, it is particularly important to distinguish between primary maldescent and secondary ascent of the testes.

Finally, in many countries, cryptorchidism is not reported as a malformation resulting in under- registration of cases.

All these reasons result in the difficulty to give a precise incidence rate of cryptorchidism.

2.2. Cryptorchidism incidence trends

As a consequence of broad variations in the definition of cryptorchidism, very few studies analyse crytorchidism incidence rate by using the same criteria.

Table 7 compares 3 cohort studies of cryptorchidism using a similar methodology for the diagnoses and follow-up: Scorer (1964) in Oxfordshire, UK, the John Radcliffe Hospital Cryptorchidism Study Group (1992) in Oxfordshire, UK (JRHCSG, 1986, 1992) and Berkowitz *et al.* (1993) in New York, USA (Berkowitz *et al.,* 1993).

Table 7. Comparison of cryptorchidism incidence rates

	Scorer (Oxford, 1964)	JRHCSG (Oxford, 1984-88)	Berkowitz (New York, 1993)
	n=3612	n=7441	n=6935
Birth	4.20	5.00	3.68
3 months	0.97	1.78	1.00
1 year	0.78	---	1.06

In Oxfordshire, between the late 1950s' and the 1980s', the cryptorchidism incidence at 3 months had doubled (from 0.97 per cent to 1.78 per cent) (JRHCSG, 1986, 1992).

A meta-analysis published in 1999 by the International Clearing House of Birth Defects Monitoring Systems (a non-governmental organization depending on the WHO) showed similar trends in several industrialized countries: in the United States and in Canada, a continuous increase has been registered and the incidence has

almost doubled over the last two decades (from 1.4 percent in 1974 to 2.4 percent in 1994). In Europe, the situation is rather similar, even if wide discrepancies are observed between countries (Paulozzi, 1999).

2.3. Risk factors for cryptorchidism: epidemiological data

Before 1995, a number of epidemiological studies were conducted in order to identify the risk factors for cryptorchidism.

Swerdlow *et al.* (1983) compared 146 cases of orchiopexy for undescended testes and 146 matched controls (Oxford Record Linkage Study) and found that the risk of cryptorchidism was higher for boys born to primiparous or young mothers, delivered in a breech presentation, and for boys born with a low birth weight for gestational age.

Depue (1984) showed in a case-control study on white male children that the risk for cryptorchidism was significantly higher in boys with low birth weight for gestational age, and in the case of administration of estrogens during pregnancy.

Hjertkvist *et al.* (1989) compared cryptorchid boys (n = 2424) with all boys born in Sweden (from the Swedish personal identification code and the Medical Birth Registry) between 1973 and 1982. According to pregnancy and delivery items, a significantly increased risk of cryptorchidism was associated with the first birth, caesarean section or toxaemia and with children small for gestational age. A seasonal (from January to March) increase in cryptorchidism incidence was also observed.

By registering cases of congenital malformations (n = 222) and healthy controls (n=443) from offspring of floriculture workers and their wives in Columbia (1982-1983), Restrepo *et al.* (1990) observed no significant difference between cryptorchid and controls according maternal exposure during pregnancy. However, the number of cryptorchid children was small (n = 16) and the list of pesticides used longer than one hundred, rendering it difficult to show differences of exposures

A case control study performed on 244 cases and 488 controls in British Columbia (Canada), found no significant association between exogenous or endogenous estrogens exposure and cryptorchidism (McBride *et al.,* 1991).

In 1995 a prospective hospital-based cohort study was conducted in New York. Seven thousand singleton male neonates (1987-1990) were enrolled in the study.

The risk of cryptorchidism was higher in children born from obese mothers, delivered by caesarean section, born preterm or with a low birth weight for gestational age, and for infants born with congenital abnormalities. A seasonal peak of cryptorchidism from September to November was also observed (Berkowitz *et al.,* 1995).

More recently, four large population case-control studies have been performed in England, Denmark, Sweden and Austria.

In England, by using the Oxford Record Linkage Study, Jones conducted a study on 1449 boys treated for cryptorchidism and 10811 random matched controls. The risk of cryptorchidism was higher for low birth weight and premature children, but also for low parity, low social class of the mother, or in case of breech presentation and pre-eclampsia during pregnancy (Jones *et al.,* 1998).

In Denmark, Weidner *et al.* (1999) compared 6177 cryptorchid boys to 23 273 male controls born alive from 1983 to 1992. The risk of cryptorchidism was associated with birth weight, independently of the duration of gestation. It was almost four times higher when an older brother had a history of the same conditions. Firstborn infants were at slightly higher risk. Finally, twins were found to have a lower risk for cryptorchidism than a singleton born in the same birth weight classes.

The same group obtained information about characteristics of mothers, pregnancy and birth, by using a mailed questionnaire sent to the mothers of 274 cryptorchid boys and 297 controls. A maternal age of 30 and above (OR = 1.9, CI = 1.2-3.0) and a low birth weight for gestational age (OR = 0.4 for 4,500 g and above, and 2.3 for 2,500 and below) impacted the cryptorchidism incidence (Moller and Skakkebaek, 1997).

In Sweden, by comparing 2782 boys undergoing surgery for cryptorchidism and 13 916 controls Akre *et al.* (1999) added to the previous factors the event of birth before the 33rd week of gestation.

In Austria, Mayr *et al.* (1999) performed a retrospective hospital based study on 447 boys undergoing orchiopexy and an equal number of healthy male age-matched controls. The frequency of cryptorchidism was higher in case of foetal growth retardation (small for gestational age), complicated deliveries, chronic diseases in relatives, and in first and second-born boys.

To summarize, as stated by Weidner *et al.* (1999), a low birth weight for gestational age, caesarean-section, breech presentation, low-parity and twin ship may be integrated into a comprehensive model to evaluate the risk. The main hypothesis is that foetal growth retardation may be due to utero-placental malfunction, and an inadequate androgen production. However, placental dysfunction cannot explain all cases of cryptorchidism (a low social class in the mother, a brother with cryptorchidism).

2.4 Molecular pathophysiology of cryptorchidism

The differential evolution during embryogenesis of 2 mesenchymatous ligaments, i.e. the cranial suspensory ligament and the Gubernaculum testis (or caudal ligament), has a crucial role in the final position of male and female gonads:

In female, persistence and development of the cranial suspensory ligament and regression of Gubernaculum testis are responsible for the intra-abdominal position of the ovaries.

In male, intrascrotal position of the testis results from regression of the cranial suspensory ligament and outgrowth of Gubernaculum testis and its migration to the scrotum.

Physiologically, testis descends in two phases (Hutson *et al.,* 1994):

1. The first phase of relative transabdominal migration occurs between the 10th and the 23rd week of gestation in humans (day 14 - 18 in mice); it is characterized by regression of the cranial suspensory ligament and Gubernaculum testis shortening. Gubernaculum testis also develops its caudal segment into the Gubernacular bulb.

2. The second inguinoscrotal phase occurs from the 26^{th} to the 35^{th} week of gestation (in mice by 20 days after birth); the Gubernaculum testis extends caudally into the scrotum and involutes, following the passage of the testis through the inguinal canal. More precisely, analysis of 142 foetal testes aged between 10 and 35 weeks of gestation, showed that all testes are descended into the scrotum before the 30th week of gestation (Sampaio and Favorito, 1998).

Several hormones and receptors are thought to influence testicular descent:

The **Müllerian inhibiting substance** (MIS) may play a central role during the transabdominal phase, by acting towards the recession of the cranial suspensory ligament and also by guiding the caudal enlargement of the Gubernaculum testis (Hutson *et al.,* 1997). However, a recent comparison of testicular descent and the level of outgrowth of Gubernaculum testis of mice deleted for MIS gene and of wild type mice, showed no significant results between groups (Bartlett *et al.,* 2002). In children, it has been observed that the MIS concentration is lower in cryptorchid children than in healthy controls ($p < 0.001$) (Yamanaka *et al.,* 1991). These results plead for the existence of interspecies differences, or of ways of substitution.

Insulin like growth factor 3 (INSL3), [also known as relaxing-like factor (RLF) and Leydig insulin-like protein (LEY I-L)] is expressed in the male embryo by Leydig cells. This signalling hormone, member of the insulin/relaxing hormone super family, is the ligand of LGR8 (activating the LGR8 receptor important in testis descent) (Kumagai *et al.,* 2002). INSL3 acts upon the gubernacular ligament to retain the gonad in the inguinal region, enabling it later to pass into the scrotum. Knockout mice have proved very useful in establishing how INSL3 mediates testicular descent: In this respect, cryptorchid phenotype of knocked out mice has been studied for either INSL3 or its receptor. Transgenic mice with targeted deletion of the INSL3 gene have bilateral cryptorchidism. Interestingly, exposure to estrogens is capable of inhibiting INSL3 expression in fetal testis.

In rat, INSL3 also seems to have a crucial role by causing normal gubernacular proliferation (Kubota *et al.,* 2002).

In humans, INSL3 /RLF appear to be good candidates for gene mutation as a cause of cryptorchidism. Mutation analysis of the 2 exon INSL3 gene in genomic DNA samples from 145 patients with a history of cryptorchid testis and 36 adult male controls identified 2 mutations (R49X and P69L) both located in the connecting peptide region of the protein, and several polymorphisms. The authors observed that the INSL3/RLF gene mutations were only present in 2 of 145 (1.4%) formerly cryptorchid patients (Tomboc *et al.,* 2000).

By analyzing the mutations of the INSL3 gene in 118 boys with cryptorchidism and 48 normal controls, a single base substitution causing an amino-acid change was identified. However, this mutation was observed in 27/118 (23%) cryptorchid and 12/48 (15%) controls; the authors concluded that mutations in the coding region of the INSL3 gene are not a common cause of human cryptorchidism (Baker *et al.,* 2002).

These results show a low prevalence of mutations of the INSL3 gene in the cryptorchid population. This pleads for a predominant inhibition of INSL3 of environmental (xeno-estrogen) origin.

Androgens are also believed to play a role by mediating testicular descent, notably during the 2nd phase of testicular descent. Although their role is incompletely known, some data on mutations of Androgen Receptor (AR) were recently emphasized.

Analysis of AR gene polymorphism was performed in 23 Swedish military conscripts with a history of cryptorchidism and 210 controls. It is established that polymorphic GAG and GGN segments regulate AR function. Median GGN lengths were significantly longer (24 vs. 23) in men with cryptorchidism compared with control subjects ($p = 0.001$), suggesting an association between GGN length and the risk of cryptorchidism (Aschim *et al.,* 2004).

Androgens are proposed to act indirectly by the genitofemoral nerve (GFN) releasing calcitonin gene-related peptide (Goh *et al.,* 1993; Ng *et al.,* 2005).

Estrogens: There are 2 Estrogen Receptors (ER), (1) ER alpha encoded by ESR1and (2) ER beta encoded by ESR2. Recent data suggest that ER alpha, rather than ER beta seems to play a role in cryptorchidism.

In a case control study of 63 Japanese patients with cryptorchidism and 47 male controls, Yoshida *et al.* (2005) reported an association of cryptorchidism with a specific haplotype of the estrogen receptor 1 (ESR1). How much such a mutation in ESR1 modifies sensitivity to estrogen signalling remains to be identified (Yoshida *et al.,* 2005*).*

A recent study of ER beta polymorphisms in 106 infertile men, 86 testis cancer patients, 51 boys with hypospadias, 23 with cryptorchidism and 186 controls found that compared with controls, infertile men had a 3 times higher heterogeneous Rsa1 AG-genotype (a ER beta silent polymorphism) associated with a 20% reduction in LH concentration. However, patients with cryptorchidism (as well as those with hypospadias and testis cancer) did not differ from controls concerning the frequency of any polymorphism (Aschim*, et al.,* 2005).

Regarding the aromatase activity, we dispose of data on the testes of stallions and a single cryptorchid horse. The latter had over-expression of aromatase resulting in an increased conversion of androgens to estrogens. These data support the concept that

estrogens may play a role in modulating the migration and development of testes across the species (Hejmej *et al.,* 2005).

Transcript of the HOXA10 gene: Male mice with a targeted deletion of the HOXA10 gene exhibit cryptorchidism. Evaluation of whether mutations of HOXA10 are associated with cryptorchidism in humans concluded that genetic alterations of HOXA10 may be present in some boys with cryptorchidism. However, HOXA10 polymorphisms also existed in normal controls. Therefore, further analysis of the function of the mutated protein is needed to elucidate what role this gene plays in testicular descent (Kolon *et al.,* 1999).

Other causes: In 94 cryptorchid children, Martinetti has found that HLA-A11 and A23 were significantly over-represented in comparison with the controls ($p=0.004$ and $p=0.02$ respectively) (Martinetti *et al.,* 1992).

Analysis of the genotype of children with terminal deletion of 10q showed cryptorchidism, or other troubles of male genital development (intersex phenotype, micropenis, and hypospadias) (Suzuki *et al.,* 1998).

A further gene which may be involved in testicular descent is that for the DNA-binding protein Desrt (Lahoud *et al.,* 2001). Whilst having the general growth retardation, mice with this gene ablated specifically show defects in the development of the male reproductive tract and are cryptorchids.

3. Impact of environment on cryptorchidism

Various xenobiotics have the ability to mimic the function of endocrine hormones (by binding to sex steroid hormones receptors), they are usually called disrupters, due to the fact that they result in an inappropriate action in time and/or in location.

Many of these hormonal disrupters are environmental pollutants.

Data available regarding the exposure-outcome relationship between environmental chemicals and the occurrence of cryptorchidism can be divided into: eco-epidemiological data from animal wildlife, laboratory experiences and clinical studies.

Eco-epidemiology is a science created in analogy to human epidemiology, which aims particularly at studying ecotoxicological effects of chemicals on ecosystems, biological communities, and populations (Bro-Rasmussen and Lokke, 1984). It also

takes into account genetic factors (for example inbreeding in isolated subpopulations) (Bowerman *et al.,* 1995; Moline *et al.,* 2000).

One example of a dramatic increase of cryptorchidism prevalence in a wildlife species has been observed in the Florida panther *(Puma concolor coryi).* This species is endangered because males have a prevalence of cryptorchidism up to 90 per cent and a low sperm count.

The main question was to know if these disorders were a consequence of inbreeding or of other origin. Results plead for an environmental contamination, regardless of the effects of inbreeding: determination of the serum estradiol levels in males and females showed no difference between both sexes, emphasizing that many male panthers have been demasculinized and feminized. Animals would be exposed to environmental xenoestrogens by their diet: the panther eats large amount of raccoon meat that have been shown to accumulate endocrine-disrupting pesticides (including pp'DDE, methoxychlor, mercury, polychlorinated biphenyls) after eating contaminated fishes (Facemire *et al.,* 1995).

In human (as in wildlife animal), the link between exposure and the occurrence of cryptorchidism is difficult to establish because of several problems:

First, *in vivo*, pesticides often have several targets, and may result in opposite end points.

Second, the dose of toxicant administrated to experimental animals is much more important than that resulting from occupational, or environmental, exposure.

Third, humans experience multiple exposures at the same time (with diet, drinking water, air pollution, occupation) or close in time (such as in agricultural occupations).

In humans, the oldest and most documented example of cryptorchidism induced by hormonal disruption is the example of diethylstilbestrol (DES). This non-steroidal estrogenic drug was administrated in women to prevent abortion complications until the seventies'. Its deleterious action in offspring has been clearly emphasized (Brackbill and Berendes, 1978; Gill *et al.,* 1979; Stillman, 1982; Wilcox *et al.,* 1995). Among the sons of women exposed to DES during pregnancy, 20.8% had epididymal cysts (vs. 4.9% in controls), 4.4% had hypospadias (vs. 1.1% in controls), 11.4% presented with cryptorchidism and hypoplastic testes (vs. 2.1% in

controls), and 1.5% had micropenis (vs. 0% in controls) (Sonnenschein and Soto, 1998).

Even if no association was clearly demonstrated between the occurrence of male genital abnormalities and the exposure to sex hormones other than DES during the first trimester of pregnancy (Raman-Wilms *et al.,* 1995), other epidemiological studies on occupational and environmental risk factors have recently pointed out environmental risks.

In Denmark, Weidner performed a case control study on 6177 cases of cryptorchidism and 23 273 controls. He observed a significant increase of risk of cryptorchidism in male offspring of women working in agriculture and horticulture (OR=1.67), but not in the case of the father working as a farmer. This study suggested that exposure to agricultural compounds during pregnancy may result in undescended testis, but the nature and the mode of action of the chemical product remained unknown (Weidner *et al.,* 1998).

In Hungary, Czeizel enrolled a population of more than 40,000 children living around a factory making vinyl chloride monomer and acrylonitrile, used for the production of stiffened plastic tubes and cartons for the packaging of margarine. He observed an increased incidence of cryptorchidism in offspring from mothers living within 25 kilometres radius (OR=8.6, CI=1.4-54.3) (Czeizel *et al.,* 1999).

In the province of Granada (South-Eastern Spain), Garcia-Rodriguez *et al.* (1996) carried out an ecology-based study on a geographical relationship between the frequency of orchidopexy and the use of pesticides. Near the Mediterranean Sea, large amounts of pesticides (endosulfan, lindane) were sprayed for intensive agriculture. In this region, farming was often a family activity, including the mother. In other areas, use of pesticides was lower, and only men worked in agriculture. The use of pesticides was divided in 4 levels (0-3). The author observed a relationship between the incidence of cryptorchidism and the level of use of pesticides confirmed by logistic regression analysis (OR = 2.32, CI=1.26-4.29, $p<0.05$).

A recent collaborative study showed that the rate of cryptorchidism was higher in Denmark than in Finland, thus suggesting that these differences may be “likely to be explained by environmental factors”. However, this observation is not supported by environmental exposure data (Boisen *et al.,* 2004).

Probably the most dramatic evidence of endocrine disrupters’ impact on testicular descent in humans comes from a recent epidemiological study from maternity

hospitals in East and West Berlin over the last 40 years (Oehme, 2002). For the years 1965-1971, similar cryptorchidism incidence rates were observed in both East and West Berlin (3-4%). Subsequently, in East Berlin where DDT-containing insecticides continued to be widely used (until German unification), this level was remained unchanged. On the contrary, in West Berlin where DDT was banned from 1972, cryptorchidism incidence rate decreased significantly (less than 1%) over the period 1972-1983.

For the period 1987-1997, the frequency of cryptorchidism was reduced to <0.5% in West Berlin, and to <2% in East Berlin. Therefore, this study showed dramatic correlation between cryptorchidism incidence rates in both East and West Berlin and the use of the known xenobiotic DDT.

We also dispose of interesting biological data about bioaccumulation in children.

By comparing the amount of 26 organochlorine compounds in adipose tissue from 18 cryptorchid children and 30 controls, Hosie *et al.* (2000) noticed that heptachloroeposide (chlorinated cyclodiene used as an insecticide) and hexachlorobenzene (chlorinated benzene used as a fungicide) were present in significantly higher concentrations in cryptorchid children ($p=0.009$ and 0.012, respectively) than in controls.

Such a phenomenon of bioaccumulation has also been stressed by Hadziselimovic *et al.* (2000). He compared estradiol levels in the placenta between newborns, with and without cryptorchidism. High levels of estradiol were found in the placentas of neonates with cryptorchidism, suggesting that there may be a similar increase in estradiol concentration in the foetal plasma during gestation.

In summary, even if a few researchers, like Ames, argue that the level of evidence is small for a deleterious effect of the contamination at low concentrations (Ames and Gold, 1997), many arguments from epidemiological as well as experimental studies plead for an impact of the environment on the descent of the testis.

3.1. Which pollutants may cause cryptorchidism?

Herein are listed only the most common chemicals that have been proven or suspected to interfere with testicular descent. However the list of potential molecules is longer and one may suppose that some of them have not been thought to have an endocrine active effect.

Primarily linked to organochlorine compounds, such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), adverse developmental and reproductive effects have been linked with exposure to phenolic derivatives such as p-phenylphenol (a rubber additive), o-phenylphenol (a disinfectant), and alkylphenols (detergents, paints, herbicides). Persistent organochlorine pollutants (POPs), including both pesticides such as DDT/DDE, were among the first industrial compounds identified in the environment. The use and production of DDT and PCBs were restricted and banned in the 1970s'. However these compounds are still the most abundant POPs in most wildlife and human samples, even if their concentrations decreased significantly over the past 30 years (Safe, 2000). Methoxychlor (MXC) developed as a substitute of DDT, also has endocrine active metabolites. Recently, other substances such as Non-oxynol (pesticide, lubricant, and cosmetics), endosulfan (insecticide), atrazine (herbicide), butylhydroxanisole (antioxidant), benzylphtalates (plasticizer) have also incriminated.

3.2. Through which mechanisms may chemicals cause cryptorchidism: the estrogen-like and anti-androgenic pathways

The effects of environmental endocrine disrupters are currently extensively investigated. The biological actions of hormones such as estrogen and testosterone are mediated by high-affinity receptor proteins located within target cells. Endocrine disrupters causing cryptorchidism can act by two main pathways: the estrogen-like and the anti-androgen action.

Estrogen-like action

Certain exogenous chemicals may bind to the estrogen receptor and mimic, or block, the actions of its natural hormone. Such compounds include some natural products, such as coumestrol and genistein, pharmaceuticals such as diethylstilbestrol, 17α-ethinylestradiol and tamoxifen, and industrial chemicals such as DDT, bisphenol A and nonylphenol (ECETOC, 1996; Schäfer *et al*., 1996). Compounds which have been shown to alter estrogen biosynthesis are cyanoketone, ketoconazole and the fungicide fenarimol (Hirsch *et al*., 1987). Methoxychlor, chlordecone (kepone), DDT, some PCB and alkylphenols can disrupt estrogen receptor function (White *et al*., 1994). O,p'-DDT and chlordecone can inhibit ligand binding to the estrogen receptors (Kelce *et al*., 1997). Various organochlorine pesticides (endosulfan, dieldrine…) are able to bind with the estrogen receptor and displace estradiol from its site on the receptor.

Table 8 gives information concerning the estrogenic potency of main endocrine disrupters.

Table 8. Estrogenic potency of main endocrine modulators

Compound	Effective concentration [Mol/l] (A)	Concentration in human serum	Relative Potency (B)	References
Estradiol	3×10^{-11}	187 ng/l (0.69 nMol/l)	1	(A) Sonnenschein *et al.,* 1995; Soto *et al.,*1995 (B) Lu *et al.,*1996
4-Nonyl-phenol	1×10^{-6}	< 1 ng/l (4.5 pMol/l)	2×10^{-7}	(A) Sonnenschein *et al.* 1995; Soto *et al.,* 1995 (B) Müller, 1997
Bisphenol A	1×10^{-6}	< 1 ng/l (4.4 pMol/l)	2×10^{-7}	(A) Sonnenschein *et al.,* 1995 (B) FDA ,1995
RCBs	1×10^{-6} -1×10^{-5}	6.7 µg/l (26 nMol/l)	1×10^{-3} -1×10^{-4}	(A) Soto *et al.,* 1995 (B) Wolff *et al.,* 1993
Dieldrin	1×10^{-5}	0.2 ng/l (0.52 pMol/l)	2×10^{-9}	(A) Soto *et al.,* 1995 (B) Anderson *et al.,* 1998
o,p'-DDT	1×10^{-5}	0.06 µg/l (0.17 nMol/l)	7×10^{-7}	(A) Soto *et al.,* 1995 (B) Anderson *et al.,* 1998
p,p'-DDT	1×10^{-5}	0.2 µg/l (0.56 nMol/l)	2×10^{-6}	(A) Soto *et al.,* 1995 (B) Göen and Angerer. 1997
Genistein	1×10^{-7}	0.006µMol/l	0.09	(A) Zava and Duwe,1997 (B) Adlercreutz *et al.,* 1993; Xu *et al.,* 1995

Studies with DES have demonstrated that administration during critical stages of genital development may induce cryptorchidism in the exposed male offspring (Newbold, 1995; Newbold *et al.,* 1986; Visser *et al.,* 1998). DES suppresses

gubernaculum outgrowth, induces production of estrogen receptors within the wolffian ducts and the stabilization of Müllerian ducts (Visser *et al.,* 1998), while also down-regulating the production of insulin-like growth factor 3 by embryonic Leydig cells.

In rodents, by exposing pregnant mice to various doses of ethinyl estradiol, Walker has demonstrated a significant and dose-dependent increase in cryptorchidism incidence in the male offspring. The author confirmed that the critical period of exposure was during the trans abdominal migration phase (Walker *et al.,* 1990).

There is also evidence for a role of modulators in bioavailability. Certain chemical compounds may displace endogenous estrogens from sex hormone-binding globulin (SHBG) sites and increase the level of bioavailable estrogens. This excess may lead to the disruption of the androgen-to-estrogen balance, and may result in cryptorchidism during the critical period of sex differentiation (Danzo, 1997; Dechaud *et al.,* 1999). SHBG variations may be involved in various situations.

The increased risk of cryptorchidism in primiparous women may be explained by the higher level of free estradiol during the first trimester of pregnancy (Depue *et al.,* 1983; Bernstein *et al.,* 1986; Bernstein *et al.,* 1988) In obese mothers as well, reduced SHBG levels are observed, and result in an increase in the free estrogen fraction (de Moor and Joossens, 1970).

Anti-androgen action

The hypothesis that cryptorchidism may result from an anti-androgen effect is based on toxicology results for certain pesticide compounds: op'DDT and its metabolite pp'DDE, vinclozoline and phthalates, that have an antiandrogen activity (Goh *et al.,* 1993; Kelce *et al.,* 1995; Kelce and Wilson, 1997; Gray *et al.,* 1999). Among the phthalates, dibutyl phthalate (DBP) and di-ethylhexyl phthalate (DEHP) have a demonstrated anti-androgenic activity in rodents, resulting in a suppression of fetal Leydig cell production, that may lead to cryptorchidism (Mylchreest *et al.,* 1998; Mylchreest *et al.,* 2000; Li *et al.,* 2000)

In rats, the administration of a non-steroidal anti-androgen, such as flutamide, during the early Gubernaculum outgrowth phase inhibits (partially or completely) testicular descent (Husmann and McPhaul, 1991; Spencer *et al.,* 1991; van der Schoot, 1992; Shono *et al.,* 1994; Kassim *et al.,* 1997; Zakaria *et al.,* 2000). However, the use of flutamide in the perinatal period fails to inhibit testicular descent in rats (Husmann and McPhaul, 1991; Spencer *et al.,* 1991). These results

indicate that testicular descent is consecutive to androgen inhibition during the brief period of gubernaculum outgrowth.

MIS (which has been described above as playing a role in the descent of the testis) has been suggested to be under androgenic regulation. The cleavage and activation of MIS have been shown to be regulated by testosterone (Kuroda *et al.,* 1991).

4. Conclusions

Cryptorchidism is a frequent malformation (> 1per cent) and a recent increase in its incidence has been reported over recent decades in North America, as well as in Europe.

To date, a low birth weight seems to be the main risk factor for cryptorchidism. There is epidemiologic evidence from many industrialized countries of an increasing trend in congenital anomalies (hypospadias, cryptorchidism), testis cancer and altered semen quality. It has been hypothesized that prenatal exposure of the male fetus to endocrine disrupting chemical would be involved.

From invertebrates to mammals, several dramatic findings produce evidence for an impact of the environment on the male genital tract. In the field of research on the understanding of the increase of genital abnormalities, eco-epidemiology yielded interesting data.

In animals, various environmental factors have demonstrated their ability to mimic, antagonise or interfere with androgens and estrogens, or to disrupt the physiological estrogen/androgen balance. However, there is limited evidence for such a phenomenon in humans.

Further research focusing on endocrine modulation is desirable, in order to ensure full validation of effects of endocrine disrupters.

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ADVANCED GLYCATION END PRODUCTS IN POLYCYSTIC OVARIAN SYNDROME

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Summary

Polycystic ovary syndrome (PCOS), a condition associated with hyperandrogenemia, hyperinsulinemia and insulin resistance, is also presented with an increased risk for atherosclerosis. Advanced glycation end products (AGE) being a diverse and heterogeneous group of compounds have been implicated in the pathogenesis of many disorders including diabetic microvascular complications, connective tissue diseases particular in rheumatoid arthritis and neurologic conditions such as Alzheimer's disease, as well as end-stage renal failure. Interestingly, it has been shown that precooked meals, overcooked food and several contemporary beverages contain increased amounts of AGEs which appeared to be absorbed by the intestinal tract. It is possible that this environmental contribution of exogenous AGEs to the endogenous pool in the general population and in particular in patients with increased endogenous AGE levels may be involved in the pathogenesis of atherogenesis or in the acceleration of its appearance. Recently, we have demonstrated increased AGE levels in women with PCOS which may be implicated in the endothelial dysfunction and infertility underlying this disorder.

The present chapter focuses on the detrimental effects of progressive AGE accumulation in the human body with particular emphasis on the pathological crosslinking of collagen formation, as well as on the endothelial dysfunction through increased oxidative stress, vasoconstriction, and creation of a procoangulant state and induction of pro-inflammatory responses. All these effects can alter the ovarian structure, its hormonal milieu and the whole reproductive function, further contributing to the infertility underlying the pathogenesis of PCOS.

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 119–140.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age and is estimated to affect 10 % of the population (Dunaif, 1992). Prominent features of the syndrome include hirsutism, menstrual dysfunction infertility, elevated androgen levels and insulin resistance (Burghen *et al.,* 1980; Dunaif *et al.,* 1987). The latter is associated with a higher risk of type 2 diabetes, altered lipoprotein profiles and disturbance of the fibrinolytic system characteristic of the insulin resistance syndrome; also known as syndrome X or the dysmetabolic syndrome (Chang *et al.,* 1983; Wild *et al.,* 1985). Women with PCOS have recently been reported to exhibit endothelial dysfunction and interestingly the magnitude of the dysfunction was related to both androgen levels, and insulin resistance (Diamanti-Kandarakis and Dunaif, 1996; Conway *et al.,* 1992).

Our recent finding of elevated Advanced Glycation End products (AGE) and their receptor in a group of young normoglycemic women with PCOS compared to age-matched normal women indicates an additional mechanism contributing to the pathogenesis of the syndrome and to metabolic and hormonal alterations (Diamanti-Kandarakis *et al.,* 2005). The fact that AGE can be administered from exogenous and environmental sources, i.e. food and smoking implicates the role of environment as an additional aggravating factor affecting the PCOS in woman's health.

The following sections provide evidence on how AGEs of endogenous or exogenous excess can account for the infertility present in these women via excessive collagen cross links formation or endothelial dysfunction.

2. Advanced glycation end products (AGE)

Advanced Glycation End products (AGE) are the late products of a chemical procedure called Maillard reaction (Figure 6), in which the carbonyl group of carbohydrates reacts non-enzymatically with primary amino groups of proteins such as lysine or arginine (Henle and Miyata, 2003; John and Lamb, 1993; Bucala and Cerami, 1992).

AGE can be formed both exogenously and endogenously. The endogenous-derived AGE is produced in the body by chemical reactions which often relate to elevated blood glucose levels. Exogenous sources are tobacco and food which are generated in the process of cooking, baking, grilling and frying (Nicholl and Bucala, 1998; O'Brien and Morrisey, 1989).

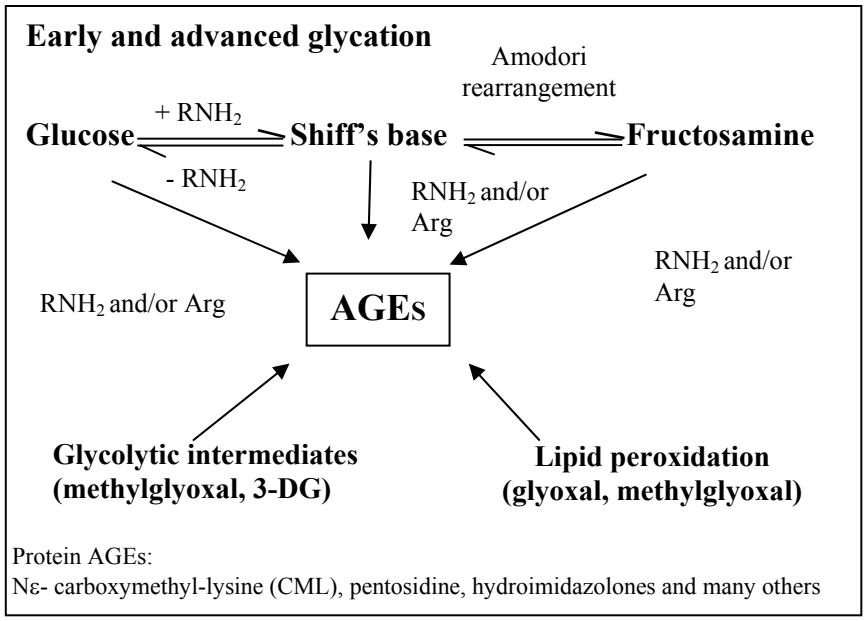
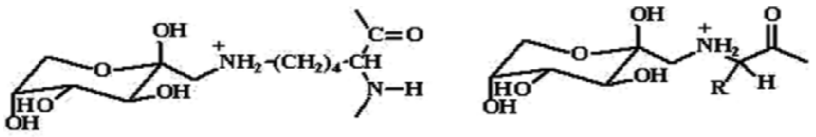


Figure 6. The Maillard reaction (Thornalley, 2005)

AGE includes a complex and heterogeneous group of compounds with diverse molecular structure and biological function (Table 9). The most well-characterised are N-ε-carboxymethyl) lysine (CML), pentosidine, 3-deoxyglucosone and methylglyoxal (Figure 7, Bierhaus *et al.,* 1998; Miyata *et al.,* 1997).

Table 9. Diversity of advanced glycation end-products (Thornalley, 2005)

A. Early glycation adducts of proteins

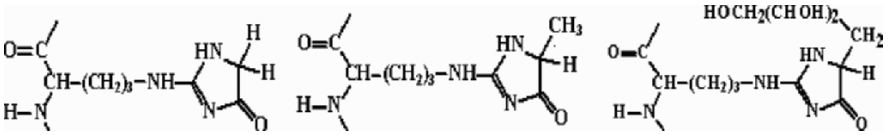


Nδ-(1-Deoxy-D-fructors-1-yl)lysine **Nα -(Deoxy-D-fructos-1-yl)amino acid**

"FRUCTOSAMINE"

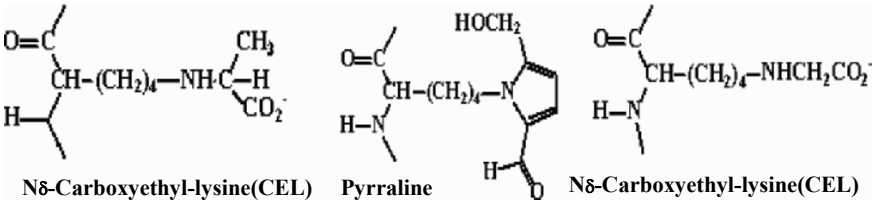
Table 9. continued

B. Protein AGEs



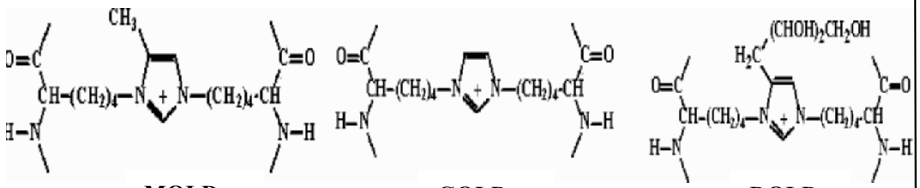
G-Himid **MG-Himid** **3DG-Himid**

Hydroimidazolones



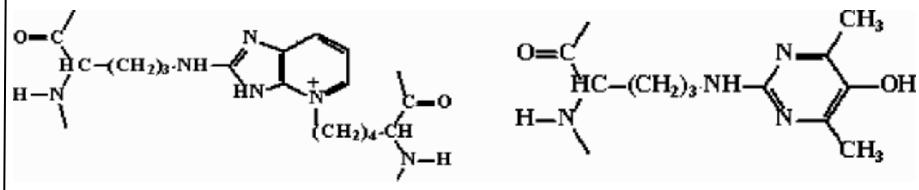
Nδ-Carboxyethyl-lysine(CEL) **Pyrraline** **Nδ-Carboxyethyl-lysine(CEL)**

Monolysyl adducts



MOLD **GOLD** **DOLD**

Bis(lysyl)imidazolium crosslinks

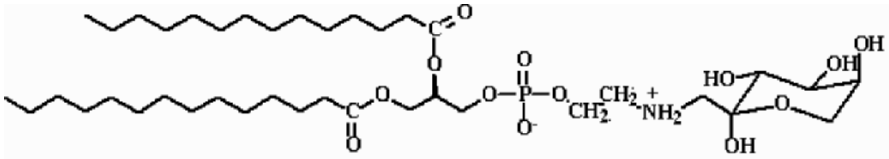


Pentosidine crosslink (fluorophore) **Argpyrimidine (fluorophore)**

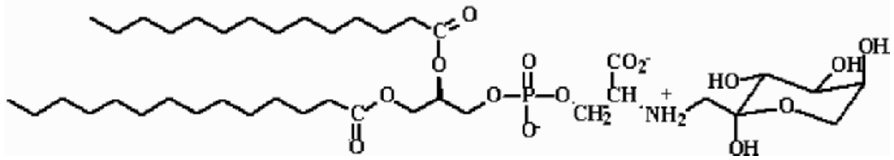
Fluorescent AGEs

Table 9. continued

C. Early glycation adducts of phospholipids

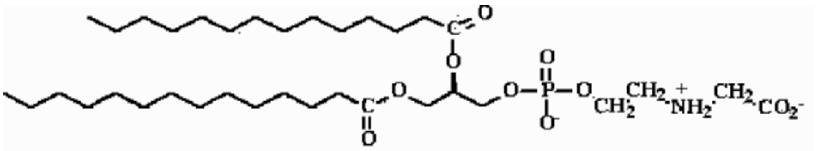


N-(1-Deoxy-D-fructos-1-yl)phosphatidylethanolamine

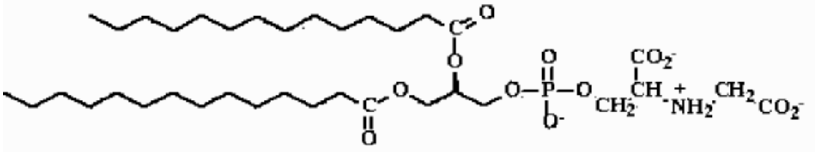


N-(1-Deoxy-D-fructos-1-yl)phosphatidlserine

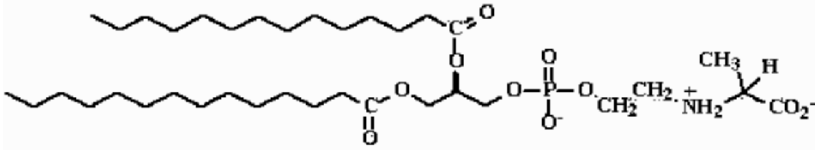
D. Phospholipid AGEs



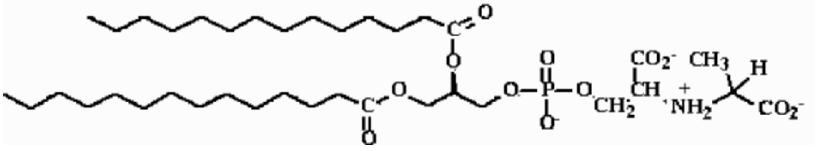
N-Carboxymethyl-phosphatidylethanolamine (CM-PE)



N-Carboxymethyl-phosphatidylserine (CM-PS)



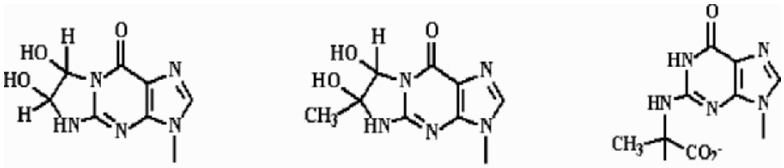
N-Carboxymethyl-phosphatidylethanolamine (CE-PE)



N-Carboxymethyl-phosphatidylserine (CE-PS)

Table 9. continued

E. Nucleotide AGEs



6,7-Dihydro-6,7dihydroxy-imidazo([2,3-b]purin-9(8)one (Gly-G)

6,7-Dihydro-6,7dihydroxy- 6methyl imidazo([2,3-b]purin-9(8)one (MG-G)

N_2-(1- Carboxyethyl) guanine CEG

A common feature is their capability of forming covalent cross-links between proteins, which is believed to be one of the main processes by which they cause damage. They have been related to the pathogenesis of many disorders including end stage renal disease, rheumatoid arthritis, cataract development, Alzheimer's disease, diabetic microvascular complications of neuropathy, retinopathy, nephropathy and many others (Masaki *et al.,* 1999; Munch *et al.,* 1997; Takahashi *et al.,* 1994; Sell and Monnier, 1990).

Proteins modified by AGE bind to cell surface receptors and other AGE binding proteins. The AGE-binding receptors are multi-ligand cell surface proteins which are expressed on a wide range of cells including smooth muscle cells, monocytes, macrophages, endothelial cells, podocytes, astrocytes and microglia Table 10 (Thornalley, 1998).

The best characterised receptors for AGE include the Scavenger Receptors types I and II, Oligosaccharyl transferase 48 (OST-48 or AGE-R1), 80K-H phosphoprotein (AGE-R2), Galectin 3 (AGE-R3) and the Receptor for Advanced Glycation End products (RAGE) (Stitt *et al.,* 1997; Schmidt *et al.,* 1996). Furthermore, lysozyme and lactoferrin have also been shown to bind AGE-modified proteins. OST-48, oligosaccharyl transferase 48; 80K-H, 80K-H phosphoprotein; Gal-3, galectin 3; ROS, reactive oxygen species (Vlassara, 2001).

The expression of some of these receptors has been found increased in some pathological conditions, including diabetes and atherosclerosis (e.g. Galectin-3 is increased in diabetic animal models and RAGE is increased in the blood vessels and kidneys of diabetic patients as opposed to control subjects, Pugliese *et al.,* 2000).

The AGE/AGE receptor hypothesis implies that degradation of extracellular AGE-modified proteins would require specific recognition by the AGE receptors,

internalization of the AGE ligand-receptor complex and proteolytic processing of the AGE-ligand (Yan *et al.,* 1994).

Table 10. AGE-receptors

Cell types	Cellular components	Soluble components	Functions
Monocyte/macrophage	AGE-R1 (OST-48)	SRAGE	Endocytosis
T-lymphocytes ($CD4^+$, $CD8^+$)	AGE-R2 (80K-H)	Lysozyme	Degradation
Endothelial cells	AGE-R3 (Gal-3)	Lysozyme $(Cx_{15\text{-}16}C)_1$	Signalling (cell activation)
Mesangial cells	RAGE	Lactoferrin $(Cx_{15\text{-}16}C)_2$	↑ ROS generation → oxidative stress
Renal tubular cells	Scavenger R (Class A)	Defensins $(Cx_{15\text{-}16}C)_n$	↑ Inflammatory response
Fibroblasts	Scavenger R (Class B) CD-36	Galectins (G1-4)	↑ Vascular permeability
Smooth muscle cells			↑ Procoagulant state
Neuronal cells			↑ Adhesion

3. Exogenous AGE

As described earlier, it is well known that during industrial processes or home cooking as well as during long-term storage of foods, lysine, and arginine may extensively become modified by Maillard reactions. With respect to this AGE formation in foods, questions arise concerning the intake of dietary AGEs via the daily food and their possible (patho) physiologic role (Henle *et al.,* 1996; Faist and Erberdobler, 2001). From the quantitative point of view, the amount of specific amino acid derivatives ingested with meals from certain heated foods fairly exceed the total amount of AGEs in the human body (Table 11, Koschinsky *et al.,* 1997). AGEs can be formed in several conditions during fermentation, cooking or just oxidation in the atmosphere (Koschinsky *et al.,* 1997).

In this context, it was proposed that serum AGE levels can be influenced by a diet containing AGEs. After eating a diet consisting of egg-white that had been heated with fructose, decrease of AGE immunoresponse in the plasma was slower in patients with renal failure compared with healthy subjects. Based on this observation, it was speculated that dietary AGEs or "glycotoxins" may represent a

risk factor in diabetic and uremic patients (Hofmann *et al.,* 2002). In recent studies, a decrease in body weight, higher plasma concentration of the AGE protein, N-ε-(carboxymethyl)lysine, and a decreased insulin response was observed in rats fed a diet high in AGEs for 20 weeks (Vlassara *et al.,* 2002). In a crossover study with diabetic patients, it was found that a high-AGE diet resulted in a slight increase in inflammatory markers such as Tumor Necrosis Factor-α and C-reactive protein (Uribarri *et al.,* 2003). A restriction of dietary AGEs in the diet for 3 days resulted in a lower AGE level in the plasma and dialysate of renal failure patients (Schwenger *et al.,* 2001). Urinary excretion of reactive intermediate Amadori products as well as of pyrraline, however, is significantly affected by food consumption and can be decreased by diets free of Maillard compounds (Foerster and Henle, 2003).

Table 11. Exogenous AGEs

Food	**AGE content (Units/100g)**
Cereal	193,400
Pastry	425,740
Cake	838,400
Duck skin	6 259,000
Condiments	**AGE (Units/15ml)**
Maple syrup	795
Soy sauce	8,700
Beverage	**AGE (Units/250ml)**
Tea	2,025
Coffee	2,200
Classic coke	8,500
Diet coke	9,500

4. AGE crosslinking and collagen

Despite the heterogeneity of AGE structures, a common consequence of their formation is covalent cross-link formation. Long-lived structural proteins such as collagen are particularly vulnerable to AGE crosslinks by nature of their slow turnover rate (Aronson, 2003). AGE crosslinking alters protein biochemistry by reducing enzymatic activity, altering biophysical properties and changing protein interactions with other enzymes (Facchiano *et al.,* 2002; Verzijl *et al.,* 2002). In the case of collagen, AGE links form throughout the molecule, contrasting the more

limited terminal positions for normal crosslinking, and this increases its tensile stiffness.

The chemistry behind crosslink formation is complex and not fully understood but is thought to involve lysine residues (Monnier *et al.,* 1996). This is supported by *in vitro* work using the agent phenacyl thiozolium bromide which can cleave chemical crosslinks between two lysine residues. Furthermore, *in vivo* studies with a synthetic thiazolidine derivative, OPB-9195 in the kidneys of Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats, a Type II (non-insulin-dependent) diabetes mellitus model, have shown prevention of the progression of diabetic nephropathy by blocking type IV collagen production and suppressing overproduction of the two growth factors, TGF-beta and VEGF (Tsuchida *et al.,* 1999).

Physiological crosslinking which also tends to involve collagen requires the enzyme lysyl oxidase (LOX). LOX catalyzes the final enzymatic reaction required for cross-linking of collagen and elastin fibers and therefore has a crucial role in regulating the formation and maintenance of extracellular matrix in the ovary. *In vivo* findings indicate control of LOX at endocrine, paracrine, and autocrine levels within the ovary and suggest coordinated regulation of ovarian extracellular matrix during follicular development, with FSH determining whether local factors act as stimulators or inhibitors of LOX (Harlow *et al.,* 2003).

The pathological crosslink formation induced by AGE leads to increased stiffness of the protein matrix, hence impeding function as well as increasing resistance to removal by proteolytic means, which in turn affects the process of tissue remodelling. These changes occur with advancing age and are accelerated in diabetes (McCance *et al.,* 1993; Paul and Bailey, 1999). Histological studies support these findings, using human aortas obtained from post-mortem examinations and showed a correlation between AGE tissue accumulation and aortic stiffness (Sims *et al.,* 1996). In addition, immunostaining methods using specific antibodies have shown increased accumulation of AGE pyrraline, crossline and pentosidine (considered to be a good biomarker of AGE crosslinking) in the kidneys of diabetic subjects (Miyata and Monnier, 1992; Furth, 1997).

Concerning the signalling mechanisms behind crosslinks, there is some involvement of the receptor for AGE (RAGE) in upregulation of type IV collagen by AGEs, a mechanism which is also believed to be involved in diabetic nephropathy (Tsuji *et al.,* 1998).

Furthermore, AGEs were found to up-regulate growth factors, such as the IGFs and transforming growth factor-beta (TGF-beta) in human and rat mesangial cells through an AGE-receptor-mediated mechanism (Table 11). The parallelism with increased extracellular matrix (ECM) deposition and altered cell growth and turnover leading to mesangial expansion raises the speculation that the enhanced synthesis of these growth factors resulting from advanced nonenzymatic glycation participates in the pathogenesis of hyperglycemia-induced mesangial expansion (Pugliese *et al.,* 1997).

Additionally, AGEs up-regulate alpha1 type IV collagen (Col4), one of the major components of ECM, through activation of the activin receptor-like kinase 1 (ALK1) in mesangial cells. This kinase was highly expressed in human diabetic nephropathy and modulation of its expression can be responsible for the initiation and progression of diabetic nephropathy (Abe *et al.,* 2004).

Also, diabetic hearts had significant increases in AGEs and elevated expression of the AGE receptors, RAGE and AGE-R3, in association with enhancement in gene and protein expression of connective tissue growth factor (CTGF, Candido *et al.,* 2003).

5. AGE crosslinking, collagen and PCOS

Increased formation and deposition of collagen characterises the stroma area in polycystic ovaries, which also contributes to dysregulation of ovarian hormonal milieu and reproductive function in these young women. A possible negative role of AGEs in the ovary as in other tissues, direct or indirect, cannot be excluded and has never been investigated in ovarian tissue from PCOS. It could also be speculated that since women with this syndrome have elevated endogenous AGEs, the environmental fortification either by food intake or smoking would have additionally detrimental effects on fertility as well as on general health issues, in women with this multifaceted disorder. Furthermore, it could be an aggravating factor contributing in increased menstrual irregularity and unexplained infertility in young women.

Additionally the exogenous AGEs received with food, may have deleterious effects on the endothelium as it happens with endogenous AGEs in diabetes, and via compromised circulation to put in danger not only the reproductive function of these women but to affect general health issues, like cardiovascular disease.

6. AGE and endothelium

The progressive accumulation of AGEs, the related overexpression of RAGE and nuclear factor (NF)-kB activation has been linked to endothelial dysfunction (Figure 7). AGE-RAGE interactions and NF-kB activation leading to oxidant stress, vasoconstriction and a procoagulant state (Singh *et al.,* 2001).

Using ELISA and immunohistochemistry AGEs have been observed in fatty streaks, atherosclerotic lesions, lipid containing smooth muscle cells and macrophages from diabetic subjects (Schleicher *et al.,* 1997; Friedman, 1999). A correlation between tissue AGEs concentrations and the severity of atherosclerotic lesions has also been shown (Stitt *et al.,* 1997).

Generation of AGEs and accumulation in the vessel wall can be due to indirect consequences of elevated blood glucose (hyperglycemia), although enhanced AGEs accumulation also occurs in euglycemia and aging, albeit to lower degrees, driven by oxidant stress and inflammation. In hyperglycemia, production of 3-deoxyglucosone, at least in part via the polyol pathway, provides an amplification loop to sustain AGE generation, oxidant stress, and vascular activation. Furthermore, recruitment of inflammatory cells bearing S100/calgranulins, also ligands for RAGE augments vascular dysfunction. It is believed that activation of RAGE is a final common pathway that transduces signals from these diverse biochemical and molecular species, leading to cardiovascular perturbation (Yan *et al*., 2003).

Interaction of RAGE with its ligands enhances receptor expression and initiates a positive feedback loop whereby receptor occupancy triggers increased RAGE expression, thereby perpetuating another wave of cellular activation. Sustained expression of RAGE by critical target cells, including endothelium, smooth muscle cells, mononuclear phagocytes, and neurons, in proximity to these ligands, sets the stage for chronic cellular activation and tissue damage.

In a model of accelerated atherosclerosis associated with diabetes in genetically manipulated mice, blockade of cell surface RAGE by infusion of a soluble, truncated form of the receptor completely suppressed enhanced formation of vascular lesions and reduced vascular permeability (Anderson *et al.,* 1999).

Amelioration of atherosclerosis in these diabetic/atherosclerotic animals by soluble RAGE occurred in the absence of changes in plasma lipids or glycemia, emphasizing the contribution of a lipid- and glycemia-independent mechanism(s) to atherogenesis, which we postulate to be an interaction of RAGE with its ligands.

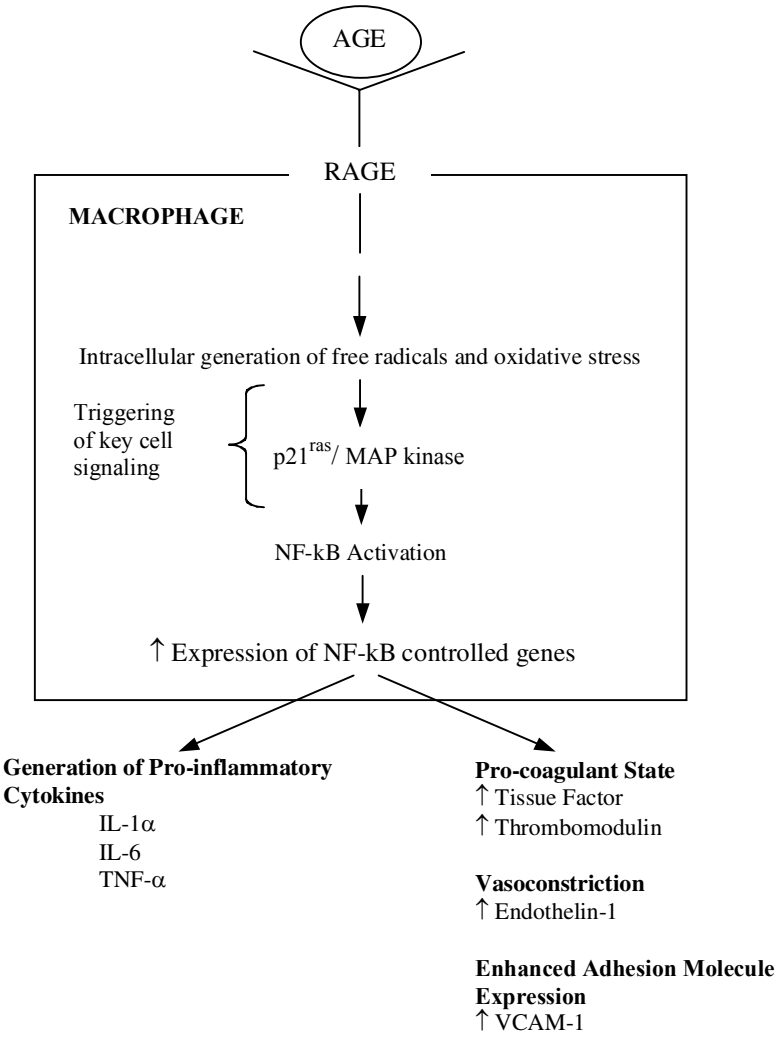


Figure 7. AGE-RAGE signalling pathways

However, sustained receptor expression in a microenvironment with a plethora of ligand makes possible prolonged receptor stimulation, suggesting that interaction of cellular RAGE with its ligands could be a factor contributing to a range of important chronic disorders (Schmidt *et al.,* 1999).

Therefore, a conserved AGE-receptor complex must be present in vascular endothelium which demonstrates subtle differences in other cell-types. In response to AGE-modified molecules, this complex is subject to upregulation, while the

AGE-R2 component also displays increased phosphorylation possibly leading to enhanced signal transduction (Stitt *et al.,* 1999).

Susceptibility to atherosclerotic lesion formation by AGE has been shown by *in vitro* studies indicating endothelial dysfunction manifested as changes in vascular permeability, coagulation and increased adherence or migration of macrophages and T-lymphocytes into the intima, with the initiation of a prolonged sub inflammatory response (Wells-Knecht *et al.,* 1996). Endothelial migration of monocytes, considered to be one of the first steps in atherogenesis, is dependent on the up-regulation of vascular cell adhesion molecule-1 (VCAM-1) expression.

AGEs have been shown to increase VCAM-1 expression by activating NF-kB (Schmidt *et al.,* 1995). Recent *in vitro* work has shown that a-lipoic acid, a natural anti-oxidant, can reduce AGE-induced endothelial expression of VCAM-1 and hence monocyte binding to the endothelium (Kunt *et al.,* 1999).

Recently, RAGE was shown to be the receptor for a novel inflammatory molecule, the high-mobility group protein-1 (HMGB1). Experiments with recombinant human HMGB1 (rhHMGB1) on human endothelial cell function by Fiuza *et al.* (2003) showed that it was capable of eliciting proinflammatory responses on endothelial cells, by increasing the expression of intercellular adhesion molecule-1 (ICAM-1), VCAM-1, and RAGE contributing to alterations in endothelial cell function in human inflammation.

Furthermore, a group of calcium-binding proteins, the S100 proteins which are putative ligands for RAGE were shown by Hsieh *et al.* (2004) to activate NF-kappaB which is a downstream regulator in RAGE-mediated transduction pathways, further promoting their reported extracellular functions, which include enhancement of neurite outgrowth, involvement in inflammation, and contribution to motility of tumour cells.

The general increase in reactive oxygen species generated from glucose-derived AGEs is among the key mechanisms implicated in tissue injury due to diabetes. AGE-rich foods could exacerbate diabetic injury, at least by raising the endogenous AGE.

Szabo *et al*. tested whether, prior to ingestion, diet-derived AGEs contain species with cell activating (TNFalpha), chemical (cross-linking) or cell oxidative properties, similar to native AGEs. By assessing the levels of glutathione (GSH) and GSH peroxidase after exposure of human umbilical vein endothelial cell (HUVECs)

to affinity-purified food-AGE extracts, they showed that food-derived AGE, prior to absorption, contain potent carbonyl species, that can induce oxidative stress and promote inflammatory signals (Cai *et al.,* 2002).

In vitro experiments in human microvascular endothelial cells and ECV304 cells showed that AGE, TNF-alpha, and 17beta-estradiol (E(2) can up-regulate RAGE mRNA and protein levels in these cells, with the mRNA stability being essentially invariant, through NF-kappaB and Sp-1, causing enhanced AGE-RAGE interactions, which would lead to an exacerbation of diabetic microvasculopathy (Tanaka *et al.,* 2000).

Low density lipoproteins can exist as oxidised LDL, glycated LDL and glycoxidated LDL. AGEs linked to lipids have been shown to initiate oxidative modification with the formation of oxidised LDL and VLDL (Hoff *et al.,* 1992; Mamo *et al.,* 1990). In diabetes a greater portion of LDL is glycated and oxidised (Brownlee, 1995; Makita *et al.,* 1996). The LDL receptor does not recognise modified LDL, which is taken up by macrophage scavenger receptor or AGE receptors resulting in lipid-laden foam cells in the arterial intima and the promotion of atherosclerosis (Sobal *et al.,* 1999). Using ELISA methods a positive correlation has been shown between arterial wall AGE and AGE modified LDL in the blood of diabetic subjects.

Under conditions of hyperglycaemia, glycation of HDL function can reduce paraoxonase activity, which is an HDL-associated ester hydrolase important for the prevention of LDL oxidation. A recent *in vitro* study (Hedrick *et al.,* 2000) has reported a 65% reduction in paraoxonase activity after glycation of HDL, as well as decreased ability of both glycated HDL and paraoxonase to prevent monocyte adhesion to aortic endothelial cells. This is an important initial event leading to development of atherosclerosis. The authors acknowledge that glycation took place under conditions of extreme hyperglycaemia (25 mmol/l glucose) and therefore might not reflect physiological conditions. They did however show a 40% reduction in paraoxonase activity in subjects with Type II diabetes and coronary artery disease compared with nondiabetic subjects with coronary heart disease ($P<0.0001$), albeit in a small number of patients.

Furthermore, there is evidence that people with a low paraoxonase activity are able to metabolize chlorpyrifos, pesticides with detrimental effects on fetal neurodevelopment. Utero exposure to these pesticides during pregnancy and early life leads to a smaller head circumference in offspring and impairment of their growth (Berkowitz *et al.,* 2004).

Recent research has shown that advanced glycation of ApoB contributes to the development of hyperlipidemia. AGE-specific receptors, expressed on vascular endothelium and mononuclear cells, may be involved in both the clearance of, and the inflammatory responses to AGEs. A study investigating the relationship between serum AGE-ApoB and AGEs in arterial tissue of older normolipidemic nondiabetic patients with occlusive atherosclerotic disease, compared with age-matched and younger asymptomatic persons has shown a positive correlation between arterial tissue AGEs and circulating AGE-ApoB suggesting a causal link between AGE modification of lipoproteins and atherosclerosis (Stitt *et al.,* 1997).

Furthermore, it has been shown that lipoprotein (a), Lp(a) – an independent risk factor for cardiovascular disease undergoes glycation in diabetic subjects. From *in vitro* studies glycation of Lp(a) attenuates fibrinolysis by inducing expression of plasminogen activator inhibitor-1 (PAI-1) and reducing expression of tissue-type-plasminogen activator.

AGEs have therefore the ability to cause platelet aggregation and fibrin stabilization, resulting in a predisposition to thrombogenesis and thereby contributing to the development and progression of diabetic vascular complications (Yamagishi *et al.,* 1998).

Erythrocyte surface AGEs can function as ligands that interact with RAGE on endothelium. The extensive contact of diabetic erythrocytes bearing surface-associated AGEs with vessel wall RAGE could be important in the development of vascular complications (Wautier *et al.,* 1994).

Research data indicate that interaction of AGEs with cellular targets, such as ECs, leads to oxidant stress resulting in changes in gene expression and other cellular properties, potentially contributing to the development of vascular lesions (Yan *et al.,* 1994).

7. Polycystic ovary syndrome

Atherogenesis in the vasculature is accelerated by changes in the dynamic equilibrium between endogenous tissue plasminogen activator and plasminogen activator inhibitor-1 (PAI-1). Increased expression of PAI-1, decreased expression of tissue plasminogen activator or both can lead to decreased fibrinolytic activity and predispose to thrombosis. Increased concentrations of insulin (and proinsulin) in the plasma raise plasma PAI-1, although the mechanisms of this effect are not known. In addition, it has been observed that basal fibrinolytic activity is decreased

in patients with type 2 diabetes; this may accelerate atherosclerosis by exposing vascular luminal wall surfaces to persistent and recurrent thrombi. Abnormalities in the vessel wall appear to contribute to the increased risk. There is also evidence that PAI-1 content is increased in atherosclerotic lesions of patients with type 2 diabetes, suggesting that interventions to reduce insulin resistance and improve glycemic control may improve the fibrinolytic response.

Clinical studies in patients with polycystic ovary syndrome demonstrated that treatment with troglitazone (Tgz), an insulin-sensitizing agent, can markedly reduce blood levels of PAI-1. There is also clinical evidence that these agents may contribute to regression of intimal medial thickness in patients with type 2 diabetes, providing further indication that antidiabetic interventions may help inhibit the progression of early atherosclerotic lesions (Sobel, 1999).

Recent studies with troglitazone and metformin have shown improvement of both hormonal profiles and insulin sensitivity. These modifications were associated with improvement of endothelial function, suggesting that insulin sensitizers could be a useful tool to reduce the risk of macrovascular disease in women with PCOS and perhaps in other insulin-resistant syndromes (Paradisi *et al.,* 2003; Diamanti-Kandarakis *et al.,* 2005). Furthermore studies with life-style modifications have shown that in women with PCOS the hormonal, metabolic and reproductive abnormalities can improve, suggesting that environmental factors such as food toxins, smoking, pollution etc play a fundamental role in unmasking any genetic predisposition (Norman *et al.,* 2003).

8. Conclusion

In conclusion, excessive endogenous or diet-derived AGEs that are absorbed into the bloodstream may represent a major source of chemically and biologically active toxins. The interaction of AGE proteins with specific receptors may exert significant reactivity in the body, accounting for functional and anatomical alterations of the vascular wall and ovaries and contributing to the infertility that characterises PCOS. On the basis of research findings presented in this chapter, appropriate measures to limit AGE intake, such as eliminating those foods or modes of cooking associated with the highest AGE content, may greatly reduce the already heavy burden of these toxins in the PCOS patient.

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OOCYTES AND OVARIAN FOLLICLES AS TARGETS OF ENDOCRINE DISRUPTERS: *CONSEQUENCES FOR REPRODUCTIVE HEALTH*

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Summary

This chapter illustrates the physiological characteristics of oocytes and ovarian follicles in relation with their possible exposure to environmental contaminants. The total amount of oocytes present in the adult ovary is established shortly before birth. As soon as the primordial follicle store is established, follicle recruitment begins and it continues without halting for the rest of life or until the ovary is depleted. Between recruitment and ovulation, the oocyte goes through a deep transformation in order to become able to sustain embryonic development. The permanent nature of the oocyte population together with its essential role in supporting embryonic development makes it a sensitive target for the adverse effects of environmental contaminants. Amongst the different environmental contaminants we focussed our analysis on a range of chemicals known as endocrine disrupters (EDs) because of their widespread diffusion and the potential hazard they represent for reproductive health. Their sites of action include the hypothalamus–hypophyseal system, resulting in disruption of the normal pattern of gonadotropin secretion, and the ovary, resulting in destruction of the oocyte. In turn, oocyte destruction can result from directly impairing oocyte viability or from indirect mechanisms involving alterations within the follicular wall. Recent work performed in our laboratory on bovine and pig, has begun to elucidate some of the cellular and molecular mechanisms involved in EDs negative effects on oocyte developmental competence. Our results indicate that EDs perturb maternal mRNA stability and disrupt the physiological remodelling of the cytoplasm, taking place during oocyte maturation.

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 141–158.

1. Introduction

In this chapter, we analyze the impact of environmental contaminants on oocytes and ovarian follicles. In particular, we will describe how the physiological characteristics of follicular formation and the oocyte role in embryonic development make them a very sensitive and disruptive target for the potential damages that the environment can inflict on reproductive health. Amongst the different kinds of environmental contaminants, we focussed our attention on the group of chemicals described as endocrine disrupters. These compounds, in fact, are widespread and represent a specific threat to reproductive health since reproductive physiology is fully under endocrine regulation.

2. Why is the oocyte an easy target?

The total amount of oocytes present in the adult ovary originates from a definite number of primordial germ cells (PGCs) that are formed in the yolk sac epithelium. These cells reach the primitive ovary after migrating through the gut mesentery and the gonadal ridges of the mesonephros of the early embryo (Byskov and Hoyer, 1994). Once PGCs have reached the developing ovary, they begin to differentiate into oogonia. The population of oogonia goes through a species-specific number of mitotic divisions until the cells enter meiosis and become oocytes (Gosden and Bownes, 1995). In humans and other large mammalian species, several rounds of mitotic divisions occur until shortly before birth.

When proliferation is concluded germ cells begin the process of meiosis that will halve the number of chromosomes in the oocyte making it aploid and therefore ready to join the other half of the genome carried by the sperm at the time of fertilization. However, oogonia, now termed primary oocytes, do not complete meiosis but progress only through the dictyate stage (Picton, 2001).

The initiation of meiosis in the oocytes coincides with the onset of folliculogenesis, the process that will lead to the formation and growth of the ovarian follicle. A single layer of flattened pregranulosa cells encloses dictyate oocytes forming the primordial follicles (Figure 8). Its structure is completed by stromal cells that will differentiate into a theca layer after follicle growth commences (Gougeon, 1996). Nests of primordial follicles are clearly recognizable in the foetal human ovaries by day 154 of gestation (Gosden and Bownes, 1995). All oocytes that do not become incorporated into primordial follicles will degenerate. Primordial follicles constitute the store of germ cells in the postnatal ovary and their numbers vary with species and age (Picton, 2001). As soon as the primordial follicle store (Figure 9) is

established, follicle recruitment begins and it continues without halting for the rest of life or until the ovary is depleted. Follicle growth is continuous, ending, in most cases, with the degeneration (atresia) of the follicle and its oocyte. After puberty, one or a small number of follicles will develop completely each cycle and ovulate a mature oocyte.

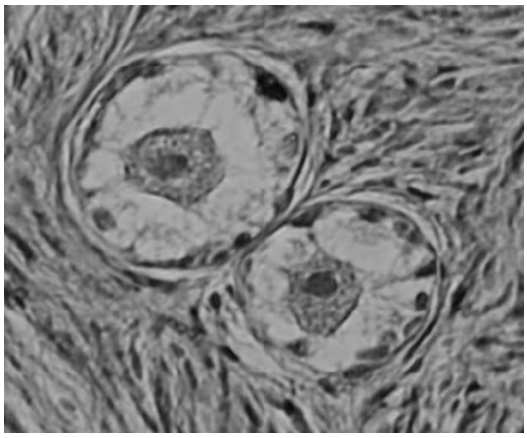

Figure 8. Primordial follicles are formed by a single layer of flattened pregranulosa cells enclosing a dictyate oocytes (original magnification 400x)

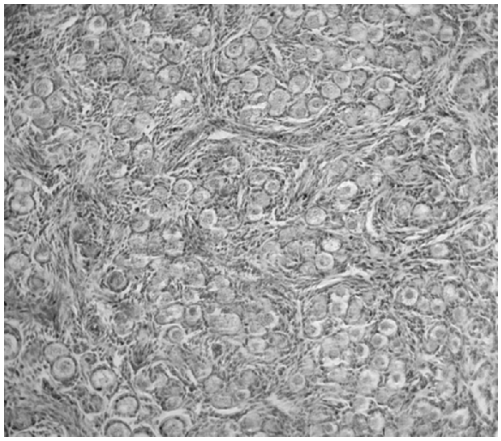

Figure 9. Primordial follicle store as it appears in the ovary of a newborn piglet (original magnification 100x)

This brief summary of oogenesis and folliculogenesis indicates that oocytes are exposed to environmental stimuli for a period that in humans can be as long as several decades. Once established, the follicular unit helps to maintain the oocyte in a controlled environment and isolates the cell from any potentially harmful

substance circulating in the bloodstream. Nevertheless, the oocyte remains a potential target for environmental insults for a much longer period than its male counterpart does. The length of spermatogenesis, in fact, is measured in months and, consequently, the effect of any non-permanent exposure to environmental contaminants can be quenched by the complete renewal of the target population. This cannot occur when oocytes are exposed because they form a permanent cell population.

3. What are the consequences of oocyte exposure?

The number of ovulated oocytes always exceeds that of born offspring since the development of a new individual is a long and complex process that can fail at any step. Each step can occur only if all the previous ones have been completed correctly. Therefore when failure of embryonic or foetal development is observed, the cause must be investigated in the preceding stages. Since fertilization is the earliest step of development, the quality of gametes is critical to generate a zygote with the highest potential to complete its development to term. The development of the conceptus to term and the quality of the gametes are closely related through a complex network of events that begins well before fertilization.

In vitro embryo production technology has allowed the study of the role played by the quality of gametes in embryonic development. Since the role of spermatozoa is extensively described elsewhere in this volume, this review deals only with the role played by the oocyte in determining the developmental potential of the embryo.

Oocyte competence is acquired gradually and increases together with follicular development. In this context, "gradual increase" refers to the percentage of competent oocytes and not to a higher competence of a single oocyte (Gandolfi, 1998). In cattle, follicles of larger diameter have been demonstrated to contain oocytes with higher developmental potential (Lonergan *et al.,* 2003). Oocyte diameter is directly proportional to the follicle diameter and the oocyte continues its growth even in follicles with a diameter >10 mm (Arlotto *et al.,* 1996). Therefore, follicle size and oocyte diameters are closely related and an increase in both improves oocyte capability to develop. This indicates that oocyte competence is acquired within the ovary during the developmental stages that precede ovulation through a process referred to as "oocyte capacitation" (Hyttel *et al.,* 1997). Though the precise mechanisms are unclear, it can be hypothesized that, during capacitation, oocytes become equipped for sustaining future embryonic development while, during maturation, an appropriate signal must be provided in order to trigger the

developmental program acquired in the previous phase (Moor and Gandolfi, 1987). These two different functional phases are likely to be regulated by distinct processes.

In particular, the oocyte role is critical during the interval between fertilization and the so-called maternal-embryonic transition (MET) when the transcriptional activity of the embryonic genome becomes fully functional. During this period, embryonic development is supported by maternal RNAs and proteins synthesized during oogenesis. The length of this period depends on the species considered. In mammals, it can occur as early as the late 2-cell stage as in the mouse or later in development as the 4-cell stage in pigs, between the 4- and 8-cell stage in human embryos, the 8-cell stage in rabbits and between the 9- and 16-cell stage in sheep and bovine embryos (Telford *et al.,* 1990).

In cattle oocytes, for example, transcriptional activity has been reported as early as the secondary follicle stage, when both heterogeneous nuclear RNA (hnRNA, the precursor of messenger RNA) and ribosomal RNA are synthesized. Such activity progresses until the oocyte reaches a diameter of 110 μm and is enclosed in a 2- to 3-mm follicle (Fair *et al.,* 1997). This transcriptional activity replenishes the cytoplasmic stores of the oocyte of messenger molecules, which will be translated until the MET occurs, and possibly, beyond, as suggested by evidence obtained in different species. In the mouse, in fact, as much as 30% of maternal mRNA is still detectable at the blastocyst stage in both trophectoderm and inner cell mass (Renard, 1998), therefore, the stability of oocyte mRNA is crucial for normal development.

Any perturbation of these delicate processes is likely to reduce the oocyte developmental competence and, therefore, to cause an arrest of embryonic development at any given stage. The interval between the action and its effect can be long making it hard to correctly interpret the results. Taken together these observations suggest that:

- It is important to examine the effects of the oocyte exposure to environmental contaminants as a possible cause of altered reproductive health;

- The consequences of such exposure must not be looked for only at the time of oocyte maturation but also at the time of early embryonic development and, possible, even at later stages.

4. Endocrine disrupters

A range of environmental chemicals known as "endocrine disrupters" (EDs) have recently raised alarming concern among the general public as well as the scientific community and policy makers due to the potential hazard they represent for reproductive functions, cell homeostasis and - more generally – health (Gandolfi *et al.,* 2002).

An endocrine disrupter is defined as an exogenous agent that interferes with the synthesis, secretion, transport, metabolism, binding, action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental processes (Colborn *et al.,* 1993). The concern for these chemicals is increased by the observation that, like the natural hormones, they are able to exert their perturbing effect on the endocrine system of animals and humans even when present in minute amounts, i.e. parts per trillion.

Currently around 60 chemicals have been identified as EDs. This heterogeneous group of molecules includes: (a) synthetic chemicals used in industry, agriculture, and consumer products; (b) synthetic chemicals used as pharmaceutical drugs; and (c) natural chemicals found in human and animal food (phytoestrogens). Aproximatively half of these compounds are substituted with halogen groups, such as chlorine and bromine, and include dioxins (PCDDs), polychlorinated biphenyls (PCBs) and organochlorine pesticides as DDT, methoxychlor, dieldrin and hexachlorcyclohexane (HCH).

Exposure to EDs can occur from a number of different sources: humans and animals can be exposed involuntarily to EDs as a result of drinking contaminated water, breathing contaminated air, ingesting food, or contacting contaminated soil.

Most of these compounds display specific properties that make them intrinsically hazardous. They have long environmental half-lives resulting in a continued increase of the global concentration in the environment. They can be detected and they may concentrate at great distances from the site they were produced, used or released. They have very low water solubility and extremely high lipid solubility, leading to their bioaccumulation in adipose tissues. Finally, many of these compounds are particularly relevant for reproductive health because they possess sex steroid activities, thereby causing endocrine disruption.

Their stability and lipid solubility has led to increased concerns for the toxic effects that they can exert on a range of biota even at extremely low levels, in particular on top-of-the-food-chain species. Moreover, high concentrations of EDs are known to be present in sewage sludge from industrial, agricultural and domestic origin, that is

spread on arable land and pasture as fertilizer (Wild and Jones, 1992), and are found in water (Abbassy *et al.,* 1999; Fingler *et al.,* 1992). Farm animals ingest these substances with food and drinking water and it is likely that the rate of ingestion will increase in the future, as growing amounts of sewage sludge are recycled onto agricultural land (Wild and Jones, 1992) with an overall increase of environmental contamination exerting adverse effects on human health.

5. Endocrine disrupters affect ovarian function in different ways

Environmental chemicals can alter ovarian function acting both upstream and downstream from the ovary itself. Moreover, when the ovary is the target, consequences depend on the stage of follicular development, and the dose and duration of exposure.

How reproductive toxicants can affect ovarian function is generally not well understood but the effects can be due to one of several possible mechanisms. Sites of action include the hypothalamus–hypophyseal system, resulting in disruption of the normal pattern of gonadotropin secretion, and the ovary, causing direct destruction of the oocyte (ovotoxicity). Disruption of any of these sites can ultimately manifest as a disruption of ovarian function, resulting in infertility (Mattison, 1993).

Oocyte destruction can result from a toxic chemical directly impairing oocyte viability. However, these mechanisms might also be indirect, involving alterations within the follicular wall (Figure 10), which compromise its ability to maintain oocyte viability (Buccione *et al.,* 1990). Extensive oocyte destruction damages ovarian follicles and, in turn, destroys steroid hormone production, which can result in ovarian failure. Therefore, oocyte destruction can, ultimately, disrupt the endocrine balance, causing a reduction in oestrogen and progesterone and an increase in FSH and LH. Finally, endocrine disrupters can affect other organs, leading indirectly to altered ovarian function, for example, through metabolic alterations that change the balance of feedback control of the hypothalamus – pituitary – ovarian system (Pocar *et al.,* 2003).

Remembering the characteristics of follicle formation and growth described at the beginning of this chapter we can understand how susceptibility of the ovaries to the different classes of agents depends on the stage of development at which exposure occurs. Compounds that extensively destroy oocytes contained in primordial and primary follicles may have a delayed effect on reproduction until recruitment of growing and antral follicles can no longer be supported (Hooser *et al.,* 1994).

Conversely, chemicals that selectively damage large growing or antral follicles generally interrupt reproductive function only temporarily because these follicles can be replaced by recruitment from the greater pool of primordial follicles. Thus, these chemicals produce a readily reversible infertility that is manifest relatively soon after exposure (Davis *et al.,* 1994).

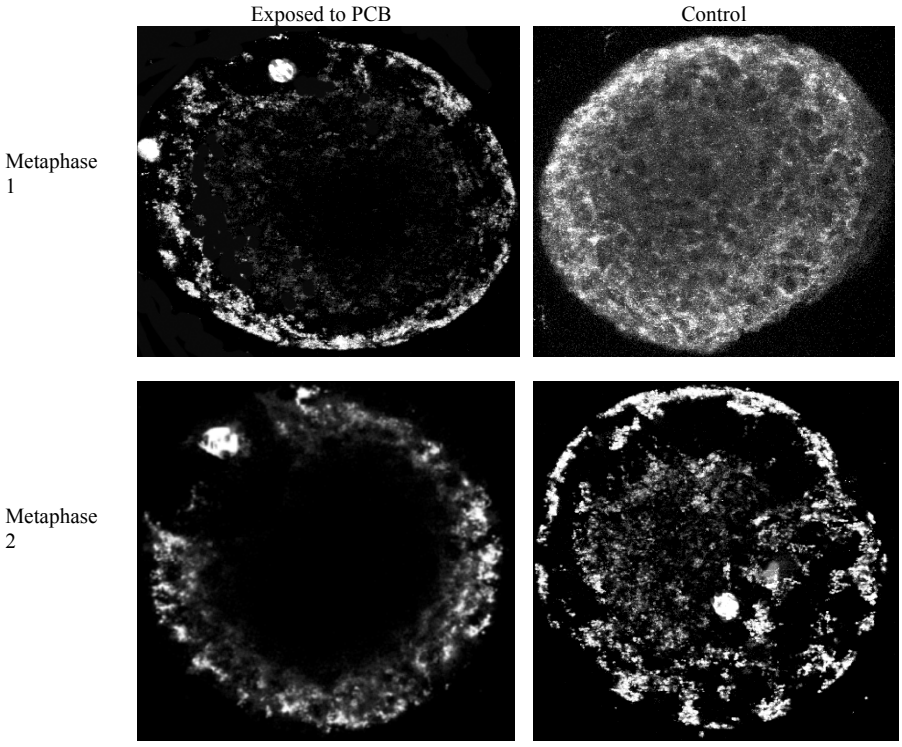

Figure 10. Pig oocytes exposed to PCB (A 1254) during *in vitro* maturation fail to form a cytoplasmic microtubule network at the time of the first metaphase. This results in an altered relocation of mitochondria and is related to a low developmental competence (Brevini *et al.,* 2004)

The ED concentration required to produce ovarian damage is another factor that determines the final effect of the exposure to a reproductive toxicant. It is only under rare circumstances that individuals are exposed acutely to toxic concentrations of ovotoxic chemicals, and the effects can usually be detected and evaluated. However, the effects of chronic exposure to toxicants are more difficult to determine. Because of the insidious nature of toxicants, this type of exposure can cause 'silent' damage and is of the greatest concern.

6. The effect of endocrine disrupters depends on the time of action

6.1. Ovarian development

The developing embryo and fetus appear to be more sensitive than adults to endocrine disruption, and alterations in reproductive development are normally observed at a concentration much lower than the one able to induce toxicity in adult animals. Rapidly dividing primordial germ cells and oogonia present during fetal development are highly sensitive to destruction by a variety of environmental chemicals (Hoyer and Sipes, 1996). This situation is aggravated by the fact that the foetus seems to be a preferential site of environmental pollutants as indicated by several observations that offspring at birth have dioxin concentrations that are up to 25% higher than maternal ones (Pocar *et al.,* 2003). Uptake of contaminants by the foetus raises concerns about the potential for adverse health outcomes. To date, few studies have investigated the effects of prenatal exposure to persistent organic pollutants (POP) on ovarian development. In utero exposure to tetrachlorodibenzo-p-dioxin (TCDD) adversely affects the reproductive function and anatomy in female rodent offspring, resulting in permanently reduced ovarian mass, decrease in the number of corpora lutea, premature ovarian senescence and early decline in fertility and fecundity (Gray *et al.,* 1997; Silbergeld and Mattison, 1987; Wolf *et al.,* 1999). Prenatal exposure of female mice to dioxin-like PCBs has been shown to reduce the number of germ cells in the ovaries by 40–50%. This decrease appears in all stages of oocytes and follicles and leads to premature reproductive ageing (Ronnback and de Rooij, 1994). No direct data are available for the effects of a pre-natal exposure to POP on the human ovary. However, in utero exposure to diethylstilbestrol, a synthetic nonsteroidal estrogen prescribed to women for medical reasons, caused infertility to their offspring due histological abnormalities of the ovary (Haney *et al.,* 1986).

6.2. Oocyte maturation

As described in detail in Section 3, oocyte maturation is a critical prerequisite for subsequent fertilization and development. Thus, disruption of this process has considerable potential to impair female reproduction. Oocyte destruction by environmental chemicals requires that these compounds reach the ovary. The presence in the human serum and follicular fluid of various organochlorine persistent chemicals such as PCBs, PCDDs and DDT was reported some time ago (Trapp *et al.,* 1984) and has been recently confirmed (Younglai *et al.,* 2002). Therefore, ovaries and follicles are exposed to environmental organochlorines as are

most other organs in the body. These studies also correlated the presence of pollutants with fertilization failure or low cleavage rates after IVF. Recent data are shedding some light on the possible mechanisms that determine infertility in women whose follicles contain organochlorine contaminants. A DDT metabolite, P,P'-DDE, synergise with FSH determining and increased aromatizing enzyme activity (Younglai *et al.,* 2004a) and increase both cytosolic Calcium oscillation and concentration in human granulosa (Younglai *et al.,* 2004b), while TCDD inhibits estradiol production by human luteinized granulosa cells down regulating the expression of cytochrome P450 17,20 lyase activity (Moran *et al.,* 2003). Taken together, these results suggest that poor fertility in women exposed to environmental contaminants is mainly caused by their action on granulosa cells rather than on the oocyte directly.

Similar results were obtained using *in vitro* experimental models: the addition of PCB mixtures (A-1254 and 1268) to the maturation medium, at concentrations ranging from 0.01 to 10 $\mu g/ml^{-1}$, affected the fertilizing capability of mouse oocytes. (Kholkute and Dukelow, 1997; Kholkute *et al.,* 1994). In another study, Pocar *et al.,* (2001a) investigated the adverse effects of exposure of bovine oocytes during the maturation process to A-1254. Concentrations as low as 0.01 µg/ml of A-1254 significantly decreases the percentage of oocytes that can reach metaphase II, reduces the fertilization ability of oocytes and causes a significant decrement in the proportion of cleaved embryos reaching the blastocyst stage. Interestingly, the minimum PCB concentration that induces the effects on fertilization and embryonic development (0.001 µg/ml) is lower than that causing the reduction of maturation rate. It is important to note that the concentration range used in these studies is comparable to that observed in the serum of non-exposed women (0.001 and 0.4 ng/g for PCBs 126 and 153, respectively) (Johansen *et al.,* 1994), which, in turn, is similar to the concentrations of persistent organic pollutants in follicular fluid (Kimbrough, 1995).

Most of the studies described in the literature evaluated the toxic effects of only one class of pollutant at a time, an experimental approach essential for understanding the mechanism of action of each substance. However, recently, an environmentally relevant mixture of more than 15 organochlorines (including PCBs, DDT, its metabolite DDE, and lindane) was used to investigate the effect of these chemicals on *in vitro* maturation of pig oocytes (Campagna *et al.,* 2001). This study has a particular ecological significance as mixtures, not individual compounds, are used industrially and have accumulated in the environment. An environmentally relevant mixture provides a better representation of conditions because it contains components that can interact with each other in an additive or non-additive fashion

(synergistic or antagonistic), and can interact with different receptors and molecular pathways. Campagna (Campagna *et al.,* 2001; Campagna *et al.,* 2002) demonstrated that exposing pig oocytes to an organochlorine mixture during *in vitro* maturation negatively affects the maturation rate, without increasing degeneration rate. Developmental competence of the exposed oocytes is also affected, reducing blastocyst rate and quality in a dose-dependent manner. Furthermore, there is a decrease in the quality and viability of cumulus cells which may account for reduced maturation and developmental competence.

7. Mechanisms of ovotoxicity

In the studies described previously, oocyte maturation was determined by examining nuclear morphology. However, this evaluation is incomplete since it does not allow the assessment of cytoplasmic maturation, a process through which immature oocytes acquire the competence to be fertilized and to sustain embryo development. Cytoplasmic maturation is characterized by ultrastructural and spatial rearrangements of the ooplasm Hyttel *et al.* (1997) as well as by chemical changes of the molecules stored therein Brevini-Gandolfi and Gandolfi (2001). For this reason, in our laboratory we examined the effects of exposure to a dose of A-1254 known to be detrimental to oocyte nuclear maturation and embryo development, on two important aspects of cytoplasmic maturation: modulation of maternal mRNA polyadenylation and cytoplasmic remodelling. We used bovine and pig oocytes as experimental models.

7.1. Alteration of mRNA stored in the ooplasm

It is generally accepted that mRNA and protein molecules synthesized during oocyte growth and maturation contribute to early development before embryonic genome activation in all species (Telford *et al.,* 1990). The storage of mRNA takes place during oocyte growth and the extent of poly(A) tail at the 3' end of the transcripts has emerged as an important regulatory element for determining stability (Richter, 1996) and represents a key regulatory step for early embryonic development (Brevini-Gandolfi *et al.,* 1999; Vassalli and Stutz, 1995). Results show that A-1254 induces changes to the polyadenylation pattern of five out of ten genes examined, indicating a perturbing effect exerted by this contaminant on the translational regulation of these transcripts (Pocar *et al.,* 2001b). Exposure of bovine oocytes to A-1254 during *in vitro* maturation induces changes in polyadenylation in a varied way: PCBs induce a more pronounced deadenylation of some of the genes that would deadenylate in control conditions (that is, glucose transporter type 1,

connexin-43 and plakophilin); however, at the same time, a longer poly(A) tail is observed at the 3'-end of connexin-32, a gene that normally re-adenylates during maturation. Finally, another pattern has been observed for heat shock protein 70, which instead of undergoing a deadenylation process as in control conditions, shows an extension of the tail at the end of *in vitro* maturation (Figure 11).

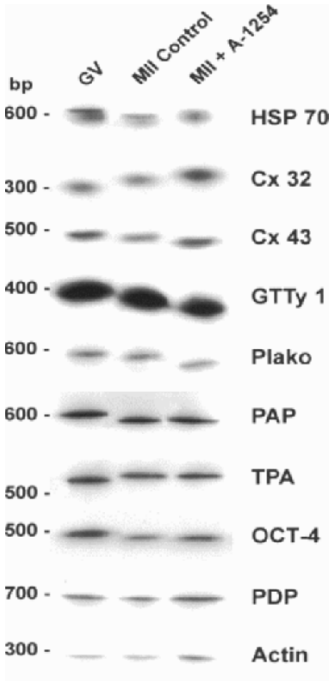

Figure 11. The exposure of bovine oocytes to PCB (A-1254) during *in vitro* maturation alters the polyadenylation levels of the mRNA molecules stored in the cytoplasm. RNA was extracted from oocytes at the time of their isolation from the follicle (germinal vesicle stage, GV) and at the end of *in vitro* maturation (second metaphase MII), which was carried out either in the absence (MII Control) or in the presence (MII.A-1254) of Aroclor at 0.1 mg/ml concentration. Left hand column shows the approximate molecular weight of each gene amplification product (bp) (Pocar *et al.,* 2001b)

7.2. Disruption of cytoplasmic remodelling

Cytoplasmic compartmentalization has been suggested to play an important role in the completion of a coordinate nuclear and cytoplasmic oocyte maturation (Combelles and Albertini, 2001). In particular, the activity and cytoplasmic distribution of mitochondria is an easily detectable marker of cytoplasm

compartmentalization. The pattern of mitochondria distribution and their metabolic activity change during oocyte maturation in many species including mouse (Calarco, 1995; Van Blerkom and Runner, 1984), cow (Stojkovic *et al.,* 2001), human (Wilding *et al.,* 2001), and pig (Sun *et al.,* 2001).

The transfer of mitochondria within different areas of the cell is mediated by a cytoskeletal network of microtubules (Van Blerkom, 1991). Microtubules, homologous polymers of α- and β-tubulin, are dynamic components of the cell cytoskeleton; they are ubiquitously present in mammalian cells and perform diverse functions such as cell shape and movement, transportation of molecules and organelles, meiosis and mitosis.

Table 12. Effect of different concentrations of Aroclor 1254 during *in vitro* oocyte maturation on meiotic resumption and parthenogenic development of pig oocytes. Data with different superscripts are statistically different as established with chi-square test ($p < 0.05$) (Brevini *et al.,* 2004)

Aroclor 1254 Concentration	Cultured oocytes	Matured oocyte (%)	Activated Oocyte	Cleaved embryos (%)	Blastocyst (%)
Control	45	43 (95.5)	193	169 (87.6)	107[a] (55.4)
0.1 ng/ml	21	19 (90.5)	34	23 (67.6)	17[a] (50.0)
1 ng/ml	21	20 (95.2)	49	38 (77.6)	27[a] (55.1)
10 ng/ml	35	33 (94.3)	108	74 (68.5)	23[b] (21.3)
100 ng/ml	24	23 (95.8)	67	51 (76.1)	14[b] (20.9)
1 μg/ml	22	21 (95.4)	57	33 (57.9)	7[c] (12.3)

We exposed pig oocytes to concentrations ranging from 0 to 1 μg/ml of A1254, during *in vitro* maturation (Table 12). This PCB mixture had no effect on maturation of pig oocytes and on the number of oocytes that cleaved following parthenogenetic activation at any of the doses tested. By contrast, a significant decrease in the number of zygotes that developed to blastocyst stage became evident at a concentration of 10 ng/ml. The number of blastocysts obtained decreased significantly, and in a dose response manner with higher concentrations. We observed a deep alteration of mitochondria relocation during maturation of pig oocytes exposed to PCBs and this was associated with the lack of a cytoplasmic microtubule network. In particular, oocyte exposed to PCBs did not show a diffused pattern of distribution of cytoplasmic microtubules suggesting that the lack of mitochondria relocation observed in these oocytes might be due to their inability to form a cytoplasmic microtubule network rather than to the inability of mitochondria

to migrate along the tubules (Brevini *et al.,* 2004). The formation of a normal meiotic spindle, observed in PCB exposed oocytes, explains why the rate of meiotic progression to the second metaphase was not altered in the presence of A1254 (Figure 10).

The latter observation illustrates clearly the hypothesis that defective oocyte developmental competence is due to the uncoupling of nuclear and cytoplasmic maturation. Indeed, while the normal formation of nucleus-associated microtubules allows the correct segregation of chromosomes during the reductive meiotic divisions, the lack of a microtubules cytoplasmic network prevents a correct relocation of mitochondria, which is likely to reflect a more generally altered compartmentalization of the ooplasm.

8. Conclusions and future directions

Data presently available clearly indicate that endocrine disrupters (singly or in combination) can affect mammalian oocyte maturation and follicle physiology even at very low concentrations. However, most of the data presently available derive from experiments performed on laboratory species or *in vitro* models; therefore, extrapolations to other species or situations should be done with caution. The lack of information on the metabolism and tissue distribution of these chemicals, which greatly depends on species physiology, concentrations and duration of exposure, as well as interactions between single components of the complex mixtures present in the environment, means that care should be taken before definite conclusions are drawn. Moreover, the specific cellular pathways activated by these compounds are still unclear. Therefore, the search for the specific mechanisms involved in ovotoxicity and metabolism of environmental contaminants should be the focus of further research in this field. In addition, future studies should systematically identify environmental chemicals that can disrupt normal development and function of the reproductive system. Currently, only about 60 environmental pollutants have been identified as endocrine disrupters and most of these have been identified accidentally, rather than because of an exhaustive screening process. Such widespread screening of all potentially toxic compounds will require the identification of appropriate biomarkers to be used for risk assessment in mammals, and the development of relevant *in vitro* markers of reproductive toxicity.

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SECTION 3:

POLLUTANTS AND EXPOSURE

NON-PESTICIDE ENDOCRINE DISRUPTERS AND REPRODUCTIVE HEALTH

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Summary

Persistent organic pollutants (POPs) include several groups of chemicals with similar structures and physical-chemical properties that elicit similar toxic effects. They have been used worldwide in agriculture (pesticides), industrial and health applications. Some POPs mimic natural hormones and are defined as endocrine disrupter compounds (EDCs), meaning that they are able to interfere with functions of the endocrine system; in fact, damage to the endocrine system is the first detectable effect. Non-pesticide EDCs are oestrogen agonists or antagonists, and as such may be responsible for the recent increase in reproductive pathologies in many species, including humans.

The link between EDC exposure and effect is not always clear or easily demonstrable; in fact the causes of health and reproductive problems in humans and wildlife are often difficult to understand, because many factors contribute to them. A link has been demonstrated, however, in accidental exposure to xenobiotics and the causation of health problems, including reproductive impairments in people exposed to them. Evidence of reproductive problems has also been reported in wildlife. Numerous experiments demonstrate the link between EDC concentration in tissues and reproductive impairment. EDC contamination is responsible for reducing sperm count and quality, female fertility and sex ratio. It is also responsible for inducing several types of cancer in both the male and female reproductive systems, and in these cases infertility may be a consequence of the cancer.

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 161–186.

1. Introduction

Persistent organic pollutants (POPs) include several groups of chemicals with similar structures and physical-chemical properties that elicit similar toxic effects. They have been used extensively worldwide in agriculture (pesticides), industrial and health applications. All these chemicals are synthetic, ubiquitous, and hydrophobic and show long-range potency (Wania and Mackay, 1993). The chemicals are persistent in soils and sediments, with environmental half-lives ranging from years to several decades, or even longer. They are not very volatile, show high chemical and thermal stability, and low biodegradability. Because of their resistance to biodegradation they are also called xenobiotics. These chemicals bioaccumulate in the lipid components of tissues in organisms, and accumulate in organisms through food webs. Consequently, the principal route for chronic exposure of both animals and humans is through diet.

Mounting evidence suggests that populations of various animal species are, or have been, adversely affected by exposure to POPs. Some xenobiotics mimic natural hormones and are defined as xenoestrogens, viz environmental chemicals that act as estrogens. Effects on the functioning of the endocrine system are the first signs of damage to be detected. In fact, some POPs are known as endocrine disrupter compounds (EDCs), meaning that they are able to interfere with functions of the endocrine system, although not all POPs are EDCs. It has been suggested that EDCs can be defined as those chemicals able 'to cause adverse effects on individual organisms through primary effects on endocrine systems that could lead to population- and community-level impacts' (Ankley *et al.,* 1997). They include the following most widespread and well-known classes of contaminants: polychlorinated-biphenyls (PCBs), -dioxins (PCDDs), -furans (PCDFs), polybrominated-diphenyl ethers (PBDEs), -biphenyls (PBBs), and other halogenated hydrocarbons.

The aim of this paper is to review the main aspects concerning the studies of the EDC effects on human and animal reproductive processes. Towards this purpose, a description of the most well-known and widespread contaminants will be given before going into the discussion at the hearth of the problem. The causes, the effects on exposed and unexposed populations (both humans and wildlife), as well as the mechanisms of actions will be reviewed through the discussion of the existing scientific literature. Lastly, results of some *in vivo* and *in vitro* experiments will also be considered.

2. Exposure to non-pesticide endocrine disrupters

2.1. Dioxins and furans

The most toxic POPs are the PCDDs and the PCDFs. They are structurally similar chlorinated hydrocarbons (Figure 12A), produced as by-products in many technical mixtures of halogenated compounds, including pesticides, and during paper and pulp bleaching (Silkworth and Brown, 1996). They also occur through urban and industrial waste incineration, metal production, fossil fuel and wood combustion

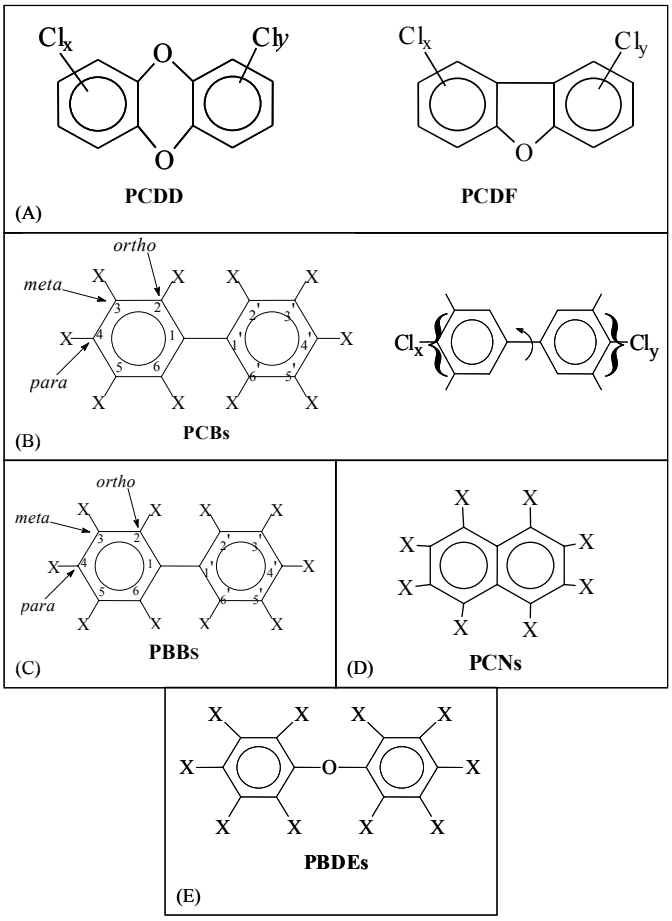

Figure 12. A-E. Chemical structure of some persistent organic pollutants (X and Y refer to the number of chlorine atoms substituted on the benzene rings).

(ATSDR, 1998), and are still present in PCB-filled electrical transformers (IARC, 1997). 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD or TCDD) is known to be the most toxic compound for organisms (WHO, 1999).

The Environmental Protection Agency (EPA) has confirmed that dioxin is a cancer hazard to people. The International Agency for Research on Cancer (IARC) announced on February 14, 1997, that the most potent dioxin, 2,3,7,8-TCDD, is now considered a Class 1 carcinogen, meaning a 'known human carcinogen' (IARC, 1997). In fact, there is evidence of carcinogenicity from epidemiological and laboratory studies in humans that have highlighted a causal relationship between exposure to TCDD and cancer (Colborn *et al.,* 1993; Mocarelli, 2004). TCDD is very persistent in the environment, but it can be slowly degraded by sunlight (ATSDR, 1998; HSDB, 2003). While it has no known commercial applications, it has been tested as a flameproofing agent and as a pesticide (ATSDR, 1998; HSDB, 2003). It also occurred as a contaminant in many technical mixtures that were widely used in the 1960s and 1970s, and as a defoliant during the Vietnamese war (Agent Orange). The toxicity of dioxins is well-documented, and many papers report the effect of exposure to TCDD and other PCDDs/Fs (e.g.: Becher and Flesch-Janys, 1998; Bertazzi *et al.,* 1998). The poisoning of Ukrainian president Viktor Yushenko in 2004 showed the world the toxic potential of this class of xenobiotics. TCDD binds with the cytosolic aryl hydrocarbon receptor (AhR) to build a substrate-receptor complex that can enter the cell nucleus and interfere with the expression of some genes (Jones *et al.,* 1985; Safe *et al.,* 1985). The model proposed for the mechanism of action of 2,3,7,8-TCDD and related toxic halogenated hydrocarbons foresees the following steps: a) the initial formation of a cytosolic receptor complex (AhR), followed by b) an activation phase; c) the formation of nuclear receptor complexes, and d) their interaction with a specific nuclear-binding site (Safe, 1990). This interaction contributes to an increase or induction of 2,3,7,8-TCDD-inducible genes, such as CYP1A1. In general, the activated dioxin-AhR complex can bind to specific sequences in the DNA, called dioxin responsive elements (DRE). The binding of the dioxin-AhR complex to the DRE causes the expression of the associated genes to be altered; this alteration in gene expression causes the toxic effects (Safe, 1990).

Estrogens induce responses through binding to the oestrogen receptor (ER), a ligand-activated transcription factor (Wormke *et al.,* 2003). The endogenous ligand for the AhR is unknown; however, this receptor binds toxic halogenated aromatic compounds, such as TCDD and other chemicals, which exhibit both AhR agonist and antagonist activities (Safe, 2001). The high affinity ligand TCDD induces several AhR-mediated changes in gene expression, tissue/species-specific toxicities,

and both tumorigenic and anticarcinogenic responses (Safe and McDougal, 2002). Wormke *et al.* (2003) reported that the inhibition of ER signalling through cross talk with the ligand - activated AhR (Safe and McDougal, 2002) and selective AhR modulators, are inhibitors of mammary tumour growth in rodents. Safe and McDougal (2002) demonstrated that TCDD inhibits oestrogen-induced responses in the rodent uterus, mammary tumors (growth inhibition) and in breast and endometrial cancer cell lines through complex inhibitory AhR-ER crosstalk.

There is a decrease in receptor-binding affinities as the lateral substitutions decrease (Safe, 1990). Toxic effects due to POPs include: cancer, reproductive and developmental problems (e.g. low birth weight, hormone alterations, lower IQ, emotional problems); alterations of the immune system, such as decreased ability to fight cancer and infections; endocrine disruption (affecting the thyroid and sex hormones); central nervous system defects, effects on the nervous system; liver damage, skin and eye disease, and finally, death (Safe, 1984; DeVito *et al.,* 1995). Apart from TCDD, many other chemicals elicit the same toxic effects, due to isostereoisomerism with TCDD. Many POPs act in the same way as TCDD and are known as "dioxin-like" compounds; these include all the PCDDs and PCDFs that have chlorine atoms in the 2,3,7,8 positions on the molecule, plus certain specific PCBs and other compounds that can be isostereoisomers of TCDD, showing AhR-mediated responses in cells, such as PBDEs (Darnerud *et al.,* 2001). Anyway, the AhR mediated activity of PBDEs is still under discussion.

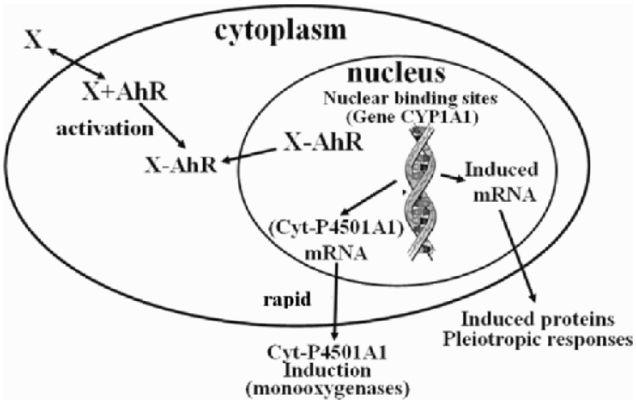

Figure 13. Mechanism of action of 2,3,7,8-TCDD and related toxic chemicals (modified after Safe, 1990)

An important tool for estimating risk in organisms is given by the 2,3,7,8-tetrachlordibenzo-p-dioxin (2,3,7,8-TCDD) toxic equivalents (TEQs); TEQs can be accumulated using the Toxic Equivalency Factors (TEFs) that express the toxic

potency of a chemical in relation to that of the 2,3,7,8-TCDD (Tanabe *et al.,* 1987; Safe *et al.,* 1990). The methods are based on the fact that dioxins cause AhR-mediated effects, and that exposure is typically due to mixtures of dioxins. TEF values are based on *in vitro* and *in vivo* induction potency of the AhR; TCDD TEF was assigned a value of one, and therefore all the other chemicals have a TEF lower than one. The EU, US-EPA and other institutions use TEFs to compare the potential toxicity of each of the individual dioxins to the relative toxicity of TCDD. The International approach was adopted by the US-EPA in 1989 (EPA, 1989). This procedure assigns I-TEFs for a total of 17 compounds. Another approach for TEFs, established by the WHO (Van den Berg *et al.,* 1998), also includes a total of 17 compounds. The WHO approach for obtaining TEF values differs from the International approach for three compounds, two of which would not significantly change any TCDD TEQ value by themselves, because of their very low TEF values. The total toxicity can be calculated as follows:

$$TEQs = \Sigma n1[PCDDi \times TEFi] + \Sigma n2[PCDFi \times TEFi] + \Sigma n3[PCBi \times TEFi],$$

where PCDDi, PCDFi and PCBi are the concentration of each congener, TEFi is the specific TEF value of each congener and Σn1, Σn2, Σn3 are the sums of the TEQ values of each class of contaminants.

2.2. Poly chlorinated biphenyls

PCBs are a family of ubiquitous contaminants which differ in the number (one to ten) and the positions of the chlorine atoms that may substitute a hydrogen atom attached to the biphenyl ring (Figure 12B). There are therefore 209 congeners grouped in ten classes of isomers that differ in their degree of chlorination. Among the 209 PCBs, a hundred congeners are used in technical mixtures and can be detected in all the environmental compartments. Because of their physico-chemical properties and their low production cost, they have many industrial applications. PCBs were banned first in the USA in the 1970s, and then in most industrialized countries, due to evidence of their widespread distribution, bioaccumulation and toxic properties. PCBs are considered to be dioxin-like compounds, since the two benzene rings are able to rotate around a carbon-carbon bond, and the molecule can thus assume a planar structure, like dioxins do (Figure 12B). Depending on the number of chlorine atoms that are bonded to the ortho positions, the PCB congener may spend a shorter, or longer time, in that configuration, thus affecting its toxicity (Safe, 1990). The congeners 3,3’,4,4’ (PCB77), 3,3’,4,4’,5 (PCB126) and 3,3’,4,4’,5,5’ (PCB169) have no chlorine atoms in the ortho positions and are

therefore called coplanar or non-ortho congeners; they are considered the most toxic congeners among PCBs (Tanabe *et al.,* 1987; Safe, 1990).

Table 13. Induction of cytochromes of the MFO system in vertebrates (Kaminsky *et al.,* 1991; Safe, 1992).

Cytochrome type	Cytochrome-inducing chemicals
P4501A (metilcolanthrene, MC-type)	• Non-ortho PCB (substitutions 3,3',4,4') • mono-ortho PCB (4,4' not substituted) • Yu-Cheng victims exposed to PCB and PCDF
P4502B (phenobarbital, PB-type)	• PCB with 2,3,6-, 2,6-, 2,5-, 2,3-, 2-, 3- substitutions • Exposed mice to Aroclor 1254 • Yu-Cheng victims exposed to PCB and PCDF • Aroclor 1016, 1242, 1254, 1260 exposed workers (capacitors, transformers)
P-448	• para and at least two meta chlorines

Coplanar PCBs should be regarded with great concern, as they have dioxin-like toxicity and are responsible for similar metabolic after-effects. They induce hepatic microsomal enzymes of the Mixed Function Oxidase (MFO) system in vertebrates. Planar PCBs with chlorine atoms in both para and at least two meta positions induce cytochrome P-450 (methylcholanthrene, MC-type), while the addition of one ortho chlorine induces cytochrome P-450 and P-448 (MC-type and phenobarbital type - PB-type); the configuration with both para and at least two meta chlorines induces cytochrome P-448 (PB-type; Kaminsky *et al.,* 1991; Safe, 1992) (Table 13). These enzyme systems convert lipophilic xenobiotics into hydrophilic compounds that can be excreted by the organism. The absence of indispensable substrates for this biotransformation leads to the formation of intermediate compounds, whose toxicity is often higher than that of the original molecule (Bergman *et al.,* 1982).

PCBs are responsible of generating oxidative stress in cells, and in particular the creation of tissue-damaging free radicals. For example, exposure to PCBs and other EDCs may cause vascular endothelial cell dysfunctions (Hennig *et al.,* 1999); in fact, the oxidant/antioxidant environment can influence the endothelial cell functions mediated by PCBs such as PCB77 (that is a MC-type cytochrome inducer) and AhR ligands (Hennig *et al.,* 1999). Henning *et al.* (1999) reported that the cellular antioxidant defense, in particular the cellular level of vitamin E, is depressed after exposure to PCBs. Vitamin E acts as an antioxidant in human blood and cell

membranes, and it can have an important role in protecting against AhR inducers damages.

PCBs have been reported to show both estrogenic and antiestrogenic activity, depending on their chlorination level (Krishnan and Safe, 1993). Some PCB congeners show estrogenic activity; other congeners become estrogenic after conversion *in vivo*, although their affinity for the receptors is lower than that for natural oestrogen (Korach *et al.,* 1987). The toxic effects of a xenobiotic compound are therefore also due to these metabolites, and include metabolic dysfunctions of the endocrine and reproductive systems, skin disorders, wasting syndrome, liver damage, teratogenesis, carcinogenesis and even death (Parkinson and Safe, 1987).

2.3. Poly chlorinated naphtalenes

PCNs are a group of seventy-five compounds based on the naphthalene ring system where chlorine atoms may substitute one to ten hydrogen atoms (Figure 12D). They have been produced and used in industry since the 1930s due to their good electrical properties, weather resistance, low flammability, high chemical and thermal stability; they are also by-products of combustion and chlorinating processes (EPA, 1975). Apart from the production and use of technical mixtures, chloroalkali plants, magnesium refineries, and waste incinerators are other sources of PCNs (EPA, 1975; Järnberg *et al.,* 1997). As PCNs are microcontaminants in PCB technical mixtures (Yamashita *et al.,* 2000), they are also released into the environment through the use of PCBs, and it is likely that they are transported together as they have almost the same physical and chemical properties (EPA, 1975). PCNs bioaccumulate in organisms, as reported by many authors studying fish, birds and mammals from different environments (e.g.: Järnberg *et al.,* 1997; Falandysz *et al.,* 1996; Nakata *et al.,* 1998; Corsolini *et al.,* 2002).

2.4. Poly brominated biphenyls

PBBs are very similar to PCBs, with bromine-atoms substituting the hydrogen atoms (Figure 12C). They show the same physico-chemical properties as PCBs. PBBs have been used as flame retardant additives in synthetic fibres, plastics and other synthetic materials. Although PBBs are not currently used in consumer products, the Consumer Product Safety Commission (CPSC) reported that hexabromobiphenyl was the primary component in the most widely-used mixture of polybrominated biphenyls (IARC, 1987).

2.5. Poly brominated-diphenyl ethers

PBDEs (Figure 12E) are a class of emerging POPs used worldwide as flame retardants (Alaee *et al.,* 2003) with a production of 67.4 ktons in 2001 (http://www.bsef.com/). Production considerably decreased in Europe following restrictions in their usage (EC, 2003). PBDEs have similar behaviour to polychlorinated biphenyls (PCBs) in aquatic and terrestrial ecosystems (Schure *et al.,* 2002), while PBDE bioaccumulation and biomagnification properties have already been reported by many authors (Boon *et al.,* 2002; de Boer *et al.,* 1998; Voorspoels *et al.,* 2003). PBDEs have also been detected in remote Arctic (Ikonomou *et al.,* 2002) and Antarctic (Corsolini *et al.,* 2005) regions that seem to be final sinks. PBDEs that show acute toxicity and prolonged exposure can affect thyroid function, can cause neurodevelopmental disorders, estrogenic and hepatic effects (de Boer *et al.,* 2000; Birnbaum and Staskal, 2004). Furthermore, a synergic effect with dioxin-like compounds or other POPs cannot be excluded.

3. Endocrine disrupters and reproductive health

All metabolic functions in organisms are regulated by the endocrine system, whose main role is to maintain the body's homeostasis. This is achieved through the secretion of molecules called hormones. The endocrine system is a collection of glands that produce hormones to regulate the body's growth, metabolism, sexual development and function. The hormones are released into the bloodstream and transported to the target tissues and organs throughout the body. Each hormone's chemical structure is specific and can be recognized by the corresponding target cells. The binding sites on the target cells are called hormone receptors, whose role is to mediate the effects of the natural hormones (Rories and Spelsberg, 1989). The maintenance of body balance and homeostasis in the body is regulated by feedback loops, or by the antagonistic action of pairs of hormones that have opposite effects on the target organs. One group of hormones are the steroid hormones, which include the sexual hormones; they have estrogenic (female sex hormones) or androgenic (male sex hormones) activity. They are cholesterol-derivates, and their chemical nature is a key point in the toxic potential of EDCs.

Some POPs bind to intracellular receptors for steroid hormones (Korach *et al.,* 1987) because of their chemical structure, provoking hormonal effects in animals (Gray *et al.,* 1989), humans (Guzelian *et al.,* 1982), and cell culture (Soto *et al.,* 1991; Soto *et al.,* 1992). They thus interfere with the functioning of receptors whose normal role is to mediate the effects of the steroid hormones (Rories and Spelsberg, 1989). An EC report has stated that an endocrine disrupter is an exogenous

substance that causes adverse health effects in an intact organism, or its progeny, subsequent to changes in endocrine functions (EC, 1997).

A xenoestrogen can act in different ways: it may be active directly through oestrogen receptors, or it may affect oestrogen metabolism (Toppari *et al.,* 1996). Different estrogenic and antiestrogenic ligands produce different complexes with the estrogen receptor. Thus, the same compound may potentially have an estrogenic or antiestrogenic effect, depending on the system and concentration. Furthermore, effects of many compounds influencing other hormone systems (e.g., antiandrogens) may mimic those of estrogens (Toppari *et al.,* 1996).

There is strong evidence that many POPs with an endocrine disrupting activity cause a range of health problems in organisms, including humans (e.g.: Colborn and Clement, 1992; Kavlock *et al.,* 1996; Ankley *et al.,* 1997; Rignell-Hydbom *et al.,* 2004; Rignell-Hydbom *et al.,* 2005). Among the non-pesticide EDCs, for example, PCBs are well known endocrine disrupters. While coplanar PCBs elicit mostly dioxin-like harmful effects, the low-chlorinated non-coplanar congeners often show an estrogenic activity. However, some high-chlorinated or coplanar congeners may show an anti-estrogenic activity, inhibiting the effect of estrogens (Kramer and Giesy, 1999).

3.1. Endocrine disrupter effects on humans

Synthetic oestrogen is a medical drug used for contraception and the treatment of diseases. It has been reported that women exposed *in utero* to estrogenic chemicals (like oestrogen agonist environmental pollutants) show a much higher incidence of cancer than unexposed individuals at the age at which reproductive organ cancers normally occur (Colborn *et al.,* 1993). The scientific literature includes many papers documenting the effects of exposure to diethylstilbestrol (DES), a xenoestrogen used by physicians to prevent spontaneous abortions in women from 1948 until 1971, after which it was banned, at least for this kind of use. Daughters whose mothers took DES during pregnancy (about 1 million or more during the 1960s and 1970s) suffered reproductive system dysfunction, abnormal pregnancies, low fertility, immune system disorders and depression (Takasugi and Bern, 1988; Hines, 1992). Vaginal clear-cell adenocarcinoma is a reproductive tract cancer that is normally found in women from their fifties onwards, but is very rare in young women; many "DES-daughters" suffered from adenocarcinomas in their twenties, when they were young adults (Herbst *et al.,* 1971).

It is now suspected that increases in the incidence of numerous reproductive abnormalities in men and women may be related to exposure to EDCs. There is mounting strong evidence that many pollutants may act as oestrogen agonists or antagonists. Non-pesticide EDCs that can mimic DES, and are thus oestrogen agonists, may be responsible for the recent increase in reproductive pathologies in many species, including humans. For example, Colborn *et al.* (1993) reviewed 'The Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans'. They reported that the clinical and experimental findings regarding DES show that consideration must be given to the following facts: breast and prostate cancer increased between 1969 and 1986 in the United States; ectopic pregnancies increased by 400 per cent in the United States between 1970 and 1987; the incidence of cryptorchidism in the United Kingdom doubled between 1970 and 1987; and sperm count has decreased by approximately fifty per cent worldwide after World War II (Colborn *et al.,* 1993). The endocrine disrupting action of pollutants in seemingly unexposed people is a subtle problem, because we are all exposed to background levels of POPs. If this level is potentially harmful, consequences for the general population could be serious, as highlighted by some researchers (Mocarelli *et al.,* 2000; Butler, 2004).

3.1.1. EDCs and infertility: mechanisms of action

The effects of EDCs on the reproductive system and fertility can be attributed to two different main mechanisms of action. The development of the reproductive system of a foetus can be affected by the endocrine disrupter effect of chemicals to which the mother is exposed during pregnancy (Aitken *et al.,* 2004). The uterine environment may therefore be responsible for alterations in the normal development of the reproductive system (e.g. malformations of sexual organs), for future alterations in hormone balance, and even for cancers. The other way that EDCs affect reproductive health is through exposure during an individual's lifespan, mainly in stages like adolescence, when the reproductive system is developing (Aitken *et al.,* 2004).

The link between exposure and effect is not always clear, or easily demonstrable. It has been suggested that some health and reproductive problems in humans may be linked to exposure to EDCs, including: testicular, prostate and female breast cancer, reduction in sperm count and quality, cryptorchidism, hypospadias, polycystic ovaries, and altered physical and mental development in children (e.g.: EC, 1997). Xenoestrogens can suppress production of the follicle-stimulating hormone (FSH) by the foetal pituitary gland. The FSH normally stimulates growth of the Sertoli cells in the developing testes; therefore, their number may decrease if FSH function

is suppressed by a xenoestrogen (Sharpe, 2003). Sertoli cells secrete different hormones during the early stages of fetal life and after puberty they also control the entry and exit of nutrients and other chemicals into the tubules of the testis. Once fully differentiated, the Sertoli cell is unable to proliferate. Therefore, a reduction in the number of these cells could have an irreversible impact on male germ-cell development (Aitken *et al.,* 2004). Aitken *et al.* (2004) also stated that we know very little about the nature of the xenobiotic-metabolizing enzymes in the male germ line, and thus the potential that chemicals have to induce genetic damage, while the relative mechanisms of action are uncertain. For example, a state of oxidative stress can be experimentally induced in testes by exposure to xenobiotics such as dioxin (Chitra and Mathur, 2004), although the biochemical mechanisms remain unclear (Aitken *et al.,* 2004). Moreover, a variation in cytochrome P450 enzymes may affect male fertility (Schuppe *et al.,* 2000).

3.1.2. Causes of health and reproductive problems

The causes of health and reproductive problems in humans are difficult to understand because many factors may contribute to the increasing incidence of tumours, as well as the reduction of sperm quality and counts. For example, a different life style may be responsible for increasing physical and mental stress, which in turn may depress the immune system and decrease the ability to fight cancer and infections. Other possible factors might be poor diet due to consumption of junk foods, and the fact that cultivated vegetables and fruits reach our tables a long time after picking: some important nutrients may be volatilised or degraded. The use of particular clothing and underwear has also been suggested to be responsible for altered male fertility: temperature changes and increased tightness of fit have all been reported to affect male fertility (Tiemessen *et al.,* 1996). It can therefore be hard to demonstrate that xenobiotics may be responsible for health and reproductive problems in humans and animals, although this has been achieved in the case of accidental exposure to xenobiotics. In fact, several industrial accidents have caused health problems, including reproductive impairments in exposed people. For example, in Japan in 1968, the Yusho poisoning occurred, when rice oil was contaminated by a PCB technical mixture, Kanechlor 400, during the production processes. More than a thousand people were affected by chloracne, eye damage, neurological and hepatic disorders and immune system alterations. Their offspring were underdeveloped and smaller than normal children, and showed slow development and abnormal teething. In 1979 a similar accident occurred in Taiwan, where people affected by the Yu-Cheng poisoning showed the same clinical effects. PCDFs and non-, mono- and di-ortho PCBs were detected in both Yu-cheng and Yusho victims (Tanabe *et al.,* 1989). Guo *et al.* (2000) studied twelve boys exposed

in utero during the Yu-Cheng poisoning; their sperm showed abnormal morphology, decreased motility and decreased capacity to penetrate hamster oocytes. The same clinical effects were detected in Yu-Cheng men exposed to PCB/PCDF after birth (Hsu *et al.,* 2003). del Rio Gomez *et al.* (2002) studied the sex ratio (the sex ratio is usually 1.06: 106 males to every hundred females) of the offspring of people exposed to the Yu-Cheng poisoning, making a comparative analysis of male and female offspring and people that were under or over twenty years of age when the poisoning occurred. Exposed men who were younger than twenty at the time of exposure had fewer male children than expected (sex ratio 0.65). No effect of exposure was seen in the sex ratio (0.93) of exposed women's offspring (del Rio Gomez *et al.,* 2002).

Mocarelli *et al.* (2000) reported a similar situation for those people exposed to dioxins when a chemical factory exploded in Seveso, Italy, in 1976. They reported that the fathers' exposure level is the best predictor of the sex of offspring, as men with high dioxin concentrations in their tissues were more likely to have daughters than sons. They studied 239 men and 296 women exposed to dioxins after the Seveso accident; their children were 346 females and 328 males showing an increased probability of female births (lower sex ratio) with increasing TCDD concentrations in the serum samples from the fathers ($p = 0.008$). The dioxin concentrations found in those fathers were similar to doses that induce epididymal impairments in rats and it is about 20 times the estimated average concentration of TCDD currently found in populations from industrialised countries (Mocarelli *et al.,* 2000). Fathers exposed when they were younger than 19 years of age sired significantly more females than males (sex ratio 0.38). These results support the hypothesis that dioxins affect the human epididymis from the time of exposure and that a lowered sex ratio persist for years after exposure (Mocarelli *et al.,* 2000). They also agree with many reports on the shifting of the sex ratio: dioxin-like compound contamination has been suggested as the cause of this shift (Moshammer and Neuberger, 2000; Rio Gomez *et al.,* 2002; James, 2002; Tiido *et al.,* 2005). del Rio Gomez *et al.* (2002) stated that the mechanism of action by which the sex ratio is altered is not yet known, but they suggested that dioxin-like compounds might have a negative effect on the viability of the Y-chromosome carrying sperm or on the development of XY-fertilised eggs. They also reported that a relationship might exist between gametes and the AhR; the induction of mutations in proteins that could lead to alterations of receptor function may alter the paths of sex determination (del Rio Gomez *et al.,* 2002). Dioxin exposure has been also associated with a delay in breast development in girls (Den Hond *et al.,* 2002).

In another accident occurring in Michigan in 1973, approximately 1900 women were contaminated by PBBs which were present as a contaminant in cattle feed: exposed women and their offspring were affected by various health and reproductive problems. PBB exposure and menstrual cycle characteristics were studied and results showed a significantly longer bleed length (increase of 0.87 days) amongst women with weight loss in the highest exposure category, compared to women with lower exposure, or without weight loss (Davis *et al.,* 2005). The time to pregnancy was studied for women who became pregnant after exposure to PBBs. A total of 478 women became pregnant after exposure to PBBs, and the time to pregnancy was retrospectively assessed for each pregnancy reported after the exposiure. The analyses revealed a slightly shorter time of pregnancy for the highest exposure group (PBB concentration ≥ 11 ppb) compared to women with exposure below the level of detection (PBB concentration ≥ 1 ppb). Multivariable analyses showed a fecundability ratio of 1:26 (0.9-1.7) for the high exposure group and 1:0 (0.8-1.2) for the middle exposure group, compared to the lowest exposure group (Marcus and Tolbert, 2002).

3.1.3. Fertility problems in men

The relationship between PCB accumulation in tissues and reduced sperm motility in men with fertility problems has been reported by Bush *et al.* (1986). More recently, Rignell-Hydbom and coll. have published a paper on exposure to PCBs and semen function; they reported that very high level of exposure to PCBs and p,p'-DDE was able to affect some human semen functions (Rignell-Hydbom *et al.,* 2004). In particular, PCB153 can affect sperm motility; although the results were not statistically significant, they are interesting because of the endocrine disrupting effects of PCB (Rignell-Hydbom *et al.,* 2004). Other studies reported a negative correlation between male semen quality and PCB exposure (e.g.: Rozati *et al.,* 2002; Dallinga *et al.,* 2002; Richthoff *et al.,* 2003). Rignell-Hydbom *et al.* (2005) reported that the presence of PCB153 in male serum was also statistically correlated with the DNA fragmentation index (DFI); this index, expressed as a percentage, represents the abnormal sperm population with DNA damage (Rignell-Hydbom *et al.,* 2005). Other authors have reported that the techniques used in these analyses give only a general indication of the DNA damage and do not provide any information on the nature, or cause, of this damage (Aitken *et al.,* 2004). Many papers report the trend towards decreasing sperm count and quality in men from various developed countries over the last few decades (Bush *et al.,* 1986; Rignell-Hydbom *et al.,* 2004, 2005); for example, a significant decrease in semen quality has been reported in France, Belgium, Denmark, and Great Britain (Kuriyama *et al.,* 2005; Toppari *et al.,* 1996). Malformations of some sexual organs (cryptorchidism, hypospadias) have

also been reported as being on the increase, as well as testicular cancer (Skakkebaek *et al.,* 2001). Testicular cancer may be related to exposure to xenoestrogens and/or antiandrogens *in utero* or during early life (Skakkebaek *et al.,* 2001).

3.2. Endocrine disrupter effects on wildlife

3.2.1. Mammals and birds

Evidence of health and reproductive problems has been reported in wildlife. Elevated concentrations of PCBs have been associated with a decrease in plasma testosterone in Dall's porpoise (Phocoenoides dalli) (Subramanian *et al.,* 1987) and PCB metabolites are responsible for a decrease in thyroid function in the common seal (Phoca vitulina) (Brouwer *et al.,* 1989). Facemire *et al.* (1995) reported that the endangered Florida panther exhibits many developmental alterations and reproductive problems, in addition to those reported for humans. While male panthers show very low sperm count and motility, a very high proportion of abnormal sperm and cryptorchidism (Toppari *et al.,* 1996), high levels of PCBs were detected in females (7.32-27.06 mg/g lipid weight) due to biomagnification (their primary food being the raccoon). Facemire *et al.* (1995) suggested that the reproductive dysfunction observed in panthers was due to the high EDC contamination of mothers. Previous studies have demonstrated that, in polluted coastal areas, PCBs affect reproductive and immune function in higher trophic predators such as marine mammals (Kannan *et al.,* 1993; Borrell *et al.,* 1996).

Regarding birds, the most exposed are seabirds and other top predators, due to the biomagnification properties of ECDs. A concentration of 210 pg/g of TEQ was recognized as the threshold level for eliciting toxicological effects in birds; in fact, Elliot *et al.* (1996) reported that the no-observed-effect-level (NOEL) in bald eagle eggs was 100 pg/g, and the low-observed-effect-level (LOEL) was 210 pg/g. A TEQ concentration of 1 ng/g is considered to decrease reproductive success in birds (Gilbertson, 1983; Kubiak *et al.,* 1989; Tillitt *et al.,* 1991). Auman *et al.* (1977) reported that a population of black-footed albatrosses nesting in such remote places like the Midway Islands suffered reproductive problems due to egg shell thinning. The healthy hatched eggs were 2.5% less than the normal hatched eggs, with a consequent decrease in reproduction success. The birds showed high TEQ concentrations in their bodies (124 pgTEQ/g).

The relationship between high TEQ values and abnormal reproductive processes and chick mortality has already been demonstrated, as reported in some papers published in the 1990s (Tillit *et al.,* 1992; Giesy *et al.,* 1994; Auman *et al.,* 1997). For

example, the correlation between TEQs, egg mortality and deformities in chicks has already been demonstrated in cormorants and terns from the Great Lakes (Giesy *et al.,* 1994). This kind of abnormality is typically related to exposure to dioxin-like compounds such as coplanar PCBs (Brunstrom, 1989; Brunstrom, 1990; Giesy *et al.,* 1994).

3.2.2. Reptiles and fish

Exposure to EDCs in the environment has been associated with decreased hatching success in snapping turtle (Bishop *et al.,* 1991). The estrogenic activity of PCBs has been tested in turtles. In these reptiles, sex determination is dictated by the environmental temperature. Eggs incubated at 26°C produce hundred per cent males, while eggs incubated at this temperature and painted with two PCB metabolites produce females (Bergeron *et al.,* 1994). If eggs are treated with both the compounds they act synergistically, producing sex reversal in eighty per cent of the eggs. It is unknown whether or not the sex reversal produces fertile adult females (Bergeron *et al.,* 1994).

With regard to fish, many papers report results regarding reproductive problems and PCB exposure through diet (e.g.: Kay *et al.,* 2005). For example, Freeman *et al.* (1982) reported that PCB exposure causes testicular steroidogenesis in the Atlantic cod. Further studies conducted by Freeman *et al.* (1984) revealed that cod exposed to PCBs *in vivo* showed an increased metabolism of steroids *in vitro* by kidney and liver tissues. Moreover, the increase in plasma testosterone concentrations associated with sexual maturity was inhibited by dietary exposure of juvenile males to PCBs. PCBs and other organochlorines have also been reported to be responsible of developmental and reproductive abnormalities in fish living in the Great Lakes of North America. For example, male coho salmon from Lake Erie were reported to show decreased fertility, lower plasma concentrations of gonadotropins and steroids, poor expression of secondary sex characteristics, and high precocious sexual maturation exhibit (Leatherland and Sonstenarg, 1982; Morrison *et al.,* 1985). Male and female reproductive problems in fish, and general EDC-induced endocrine abnormalities in fish were reviewed by Hester and Harrison (1999).

3.3. In vitro and in vivo experiments

In addition to the study of effects in exposed and unexposed populations, many papers have been published on *in vitro* and *in vivo* experiments, and have essentially confirmed the observations in wildlife. For example, Kuriyama *et al.* (2005) reported the interesting results of an experiment conducted on rats in order to study

the effects of a penta-BDE congener on neurobehavioral and male reproductive health in offspring. Rats were exposed to a single dose of BDE99 (60 or 300 μg/kg body weight on gestation day 6). BDE99 interferes with thyroid hormone homeostasis and shows neurobehavioral toxicity (Hallgren and Darnerund, 2002). Results showed that exposure to a low dose of BDE99 during development causes persistent neurobehavioral effects, permanently affecting the reproductive functions of adult males (Kuriyama *et al.,* 2005).

Experiments with PCBs highlighted that they can alter avian reproductive behavior. Giesy *et al.* (2003) reported that adult breeder doves that were fed with a PCB mixture showed aberrant incubation and courtship behaviors; PCB-dosed females were particularly affected in the later experiment, performing only a small number of courtship behaviors, resulting in a severe impairment of reproductive success. Fang *et al.* (2002) described the effects of a PCB mixture on numbers and histomorphological changes of primordial germ cells (PGCs) in gonadal regions of Day 5 Hyline chicken embryos. They reported that the PGC numbers in the gonads decreased, while the index of gonadal lesions increased, therefore, it could be suspected that the reproductive function was compromised. The adverse effects of PCBs on chicken gonadal and germ cell development were initiated during the early stages of incubation through direct toxic effects, rather than through oestrogen-mimicking actions (Fang *et al.,* 2002).

4. Conclusions

There is mounting evidence that the reproductive potential of humans and other wildlife species has been decreasing over the last few decades. Many papers published in scientific journals have reported the results of observations in wildlife and humans and of *in vitro* experiments. The action of endocrine disrupters on health, and on the reproductive system in particular, has been demonstrated in many cases regarding both humans and wildlife. Numerous experiments have been carried out to demonstrate the link between EDC concentration in tissues and reproductive impairment. However, there are still many uncertainties regarding the linkage between EDC and health problems, which are often due to the difficulties in rigorously interpreting the data. Yet there is some evidence that can not be denied: EDC contamination is responsible for reducing sperm count and quality, as well as female fertility (Aitken *et al.,* 2004), while also inducing several types of cancer in both the male and female reproductive systems. In such cases, infertility may be a consequence of the cancer.

In most developed countries, 3–6% of children are now born by assisted conception. There may be various reasons why people resort to this method, such as life style, but many of them are related to infertility. Reproduction is the key to life and the main aim of all organisms is to convey their own genes to their offspring. It is difficult to think that a general reduction in fertility is natural. It must therefore be a consequence of something wrong in the environment that is not biologically acceptable. EDCs affect every aspect of our lives: reproduction, germ-cell formation, conception, the development of organs and health in general. EDC exposure during development of the foetus and of children from birth to maturity causes permanent damage (Colborn *et al.,* 1993) that can decrease quality of life to a greater or lesser extent in both wildlife and humans. EDC exposure after maturity does not seem to permanently affect the endocrine system, but giving xenoestrogens to animals causes permanent alterations in many organs, including reproductive ones (Colborn *et al.,* 1993). It is difficult for studies carried out in the general population to scientifically demonstrate the relationship between EDC accumulation and reproductive problems. It is now very important to understand these mechanisms in depth, but it is even more important to test chemicals before emission: the reduction of emissions could represent a challenge or a distant utopia for the future. Another aspect that cannot be neglected is the evaluation of the synergic effects that can result from the interaction between EDCs, other contaminants and life style aspects.

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MEDICAL DRUGS IMPAIRING FERTILITY

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Summary

The human being is one of the least fertile among the mammals, despite the alarming scene of global overpopulation. Human fertility is certainly on the decline. There are several historical, demographic, geographic, social, genetic and environmental factors contributing to the decline. Medical drugs do contribute to a decline in the fertility of some couples. Iatrogenic causes of medical illness are increasing the world over. The chapter gives an overview of medical drugs impairing fertility in men and women. Drugs may impair male fertility by interfering with spermatogenesis, sperm motility, or by interfering with the fertilising capacity of spermatozoa. Female fertility may be compromised by impairing oogenesis, ovulation, or through the suppression of ovarian function. The incidence of acid peptic disease and hypertension is increasing globally, even amongst young men and women. Drugs used to treat these conditions impair sexual function and fertility. Several drugs used to treat connective tissue diseases affect gonadal function. Many childhood/early adulthood malignancies are either treatable or curable today. Chemotherapeutic agents, while very effective in managing malignancies, often lead to impairment of fertility. Use, and abuse, of antibiotics and antibacterials have increased phenomenally. These drugs can impair fertility, albeit temporarily. Several

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 187–205.

drugs used to treat mental illness impair sexual function and fertility. Fertility impaired by medical drugs is often reversible except when associated with antimitotic drugs. Judicious use of drugs, and use of alternate drugs if necessary, would help in the restoration of sexual function and fertility in many couples.

1. Introduction

Fertility is the cherished desire of most human beings. The susceptibility of the human reproductive system to hazardous toxic substances has been recognised since antiquity. The harmful effect of lead on human fertility has been known and documented since Roman days. Drugs and environmental chemicals/toxins adversely affect the reproductive system in both men and women.

The effect of several medications on human fertility is poorly documented. Drugs are often prescribed without the knowledge and/or the sensitivity of their effects on sexuality and fertility.

Fertility is a complex phenomenon, involving the interaction of two individuals and their gametes. While male fertility requires the need to be able to perform, besides gametes capable of fertilisation, female fertility requires, (besides gametes capable of fertilisation), a favourable uterine environment. Drugs, which impair sexual performance may in turn also, impair fertility. Several drugs can interfere with this delicate balance between sexuality and fertility, impairing fertility.

Drugs impairing sexual function have not been well documented. The patients may not be aware of, or complain about, the side effects on fertility and sexual performance of the drug. Many illnesses for which drugs are used can themselves cause infertility or sexual impairment. Some of these drugs may cause such subtle impairment that there may be no obvious physical sign.

The aim of this article is to give an overview of the medical drugs impairing fertility. The chapter outlines the drugs impairing sexuality and fertility, their possible mechanism, methods of preventing drug-induced damage and measures to conserve the fertility of people being exposed to gonadotoxic drugs.

1.1. Physiology of fertility

Male fertility requires normal male karyotype, normal hypothalamo-pituitary axis, normally descended and functioning testes, patent outflow tract and the ability to

perform sexual intercourse and deposit the semen in the posterior fornix of the vagina.

Female fertility requires normal female karyotype, normal hypothalamo-pituitary axis, and normally functioning ovaries, patent and functional tubes and a favourable uterine environment.

1.2. Physiology of male fertility

Normal spermatogenesis, erection, intromission and ejaculation are the essential steps for normal *in vivo* fertility of a man. All these processes require a complex interaction between hormones, chemical messengers, cytokines and neurotransmitters. Any interference with this delicate mechanism can lead to impairment of sexuality and fertility.

Spermatogonia originate from the primordial germ cells, which migrate from the ventral wall of the yolk sac to the genital ridge. Spermatogonia undergo a series of transformations in the seminiferous tubule under the influence of FSH and intra testicular/ seminiferous tubular testosterone, to form the spermatozoa. (Mehta and Kumar, 1999). The intratubular concentration of testosterone is 50 times the plasma levels, and is aided by active transport of testosterone from the plasma into the seminiferous tubule by androgen binding protein secreted by the Sertoli cells. This intra luminal concentration cannot be mimicked by pharmacological therapy, and testosterone therapy is more likely to produce suppression of spermatogenesis and even azoospermia (Pandiyan, 2000), through suppression of LH and FSH, than aid in spermatogenesis. Spermatogenesis takes about 72 days in the human male. A healthy man in the reproductive age group produces about 1000 spermatozoa per second (which amounts to about 86 million spermatozoa per day). The spermatozoa motility is influenced by the structure of the sperm tail, and the dynein arms. The delicate and complex process of spermatogenesis can be interfered with by several drugs and chemicals.

In most men with infertility a clear cause is not clearly discernible. Genetic causes contribute to subfertility in a substantial proportion of men (Pandiyan and Jequier 1996). The exact incidence of drug impaired infertility is not known.

1.3. Physiology of female fertility

The reproductive organs of the female comprises the ovaries, fallopian tubes, the uterus and the vagina. Pulsatile release of GnRH from the arcuate nucleus of the hypothalamus, and synchronous release of FSH and LH from the pituitary, are essential for the orderly growth of the ovarian follicle and the release of a mature oocyte. Normal ovarian endocrine function with adequate oestrogen production is essential for the development of the endometrium and the production of adequate progesterone, essential for the preparation of the endometrium for nidation.

Drugs may interfere with the orderly and synchronous release of LH, FSH, action of oestrogens on the endometrium, or interfere with the effect of progesterone on the endometrium, which can lead to impairment of fertility.

1.4. In utero exposure to drugs and impairment of fertility

Male fertility has generally been considered to be declining by several authors due to various reasons (Carlsen *et al.,* 1992; Auger *et al.*, 1995).

Genetic factors like non-pairing Y chromosome, lack of sperm competitiveness, environmental exposure to oestrogens, oestrogen-like substances and other endocrine disrupting chemicals, may all be contributing to the decline observed.

In utero exposure of a male fetus to diethylstilbestrol may lead to developmental defects in the male genital tract, and to male infertility. These children could have epididymal cysts, undescended testes, hypospadias, low sperm concentration and increased spermatozoa abnormalities (Gill *et al.*, 1979; Driscoll and Taylor, 1980; Leary *et al.,* 1984).

It has even been suggested that the underlying cause of male infertility, and several human male reproductive disorders, is often of foetal origin.

In utero exposure to nicotine has been reported to produce reduced sperm quality and increased semen abnormalities. Maternal smoking may have long-term implications for the reproductive health of the offspring. A dose-dependent association between antenatal tobacco exposure and lower sperm concentration has been observed.

Men exposed prenatally to polychlorinated biphenyls and polychlorinated dibenzofurans have an increased risk of an abnormal semen picture, characterised by

low sperm motility, increased abnormal spermatozoa and lower fertilisation rate in *in vitro* studies.

Exposure to diethylstilbestrol *in utero* has been documented to produce several developmental abnormalities in the female genital tract and also leads to serious impairment of a woman's fertility and fecundity. Abnormalities in the cervix and uterine cavity have been clearly documented (Kaufman *et al.,* 1986). The pregnancy outcome of these women has been rather poor (Decherney and Naftolin, 1988). These women have a higher risk of ectopic pregnancy, abortion and preterm birth (Barnes *et al.,* 1980). Even in women undergoing IVF, the implantation and pregnancy outcome are impaired (Karande *et al.,* 1990).

In utero exposure to diethylstilbestrol increases the risk of clear cell vaginal adenocarcinoma in women (Robboy *et al.,* 1981). There is no clear association between *in utero* exposure to diethylstilbestrol and the risk of male genital tract malignancy (Brown *et al.,* 1986).

2. Drugs impairing female fertility

High-dose oestrogen treatment in adolescence seems to reduce female fertility in later life. Oestrogen treatment has been used to reduce the adult height of tall girls (Venn *et al.,* 2004).

Recreational drugs like alcohol, nicotine, marijuana, and caffeine impair female fertility. Smoking (Nicotine) reduces a woman's fertility and fecundity. Women who smoked took longer to conceive, and this delay was dose related. The delay in conception occurred in both active and passive smokers. High alcohol consumption has been associated with increased risk of infertility.

Caffeine intake of greater than 250mg/day (more than 2 cups per day) has been associated with decrease in fertility. Non-smoking women who wish to achieve a pregnancy have been advised to reduce their caffeine intake.

2.1. Drugs impairing ovarian function

Amongst the drugs impairing ovarian function, cancer chemotherapeutic drugs rank first, and are easily the most toxic. The risk of ovarian impairment increases with age and the drug/drugs administered (Howell and Shalet, 1998). Alkylating agents produce greater ovarian impairment than other agents do (Byrne *et al.,* 1992).

Younger patients run a smaller risk of ovarian failure than women do in the reproductive age group (Larsen *et al.,* 2003).

2.2. Drugs impairing ovulation

Non-steroidal anti-inflammatory drugs and Cox–2 inhibitors interfere with ovulation and follicle rupture, leading to reversible infertility. These drugs interfere with prostaglandin-mediated inflammatory response essential for ovulation (Mendonca *et al.,* 2000).

2.3. Drugs affecting luteal function

Though there are no specific luteolytic agents, hyperprolactinaemia has been documented to interfere with luteal function.(Rosato and Garofolo, 2002). Drugs causing hyperprolactinaemia may impair luteal function and cause infertility.

2.4. Drugs affecting endometrial development

Anti-oestrogens, like clomiphene, have been widely used in the management of infertility and other gynaecological disorders. While they are useful in the management of these conditions, they are inimical to the development of endometrium, and can cause infertility, despite normal ovulation.

2.5. Drugs interfering with implantation

Human implantation is a complex, highly selective, closely regulated process, requiring the constant and continuous interaction between the embryo and the endometrium.

Drugs like RU 486, anti-progestins, emergency contraceptive pills, mini pill or progesterone-only pill, interfere with implantation. These drugs are, of course, primarily used for contraceptive purposes.

2.6. Drugs impairing tubal functions

There are no specific drugs impairing tubal function. Prostaglandin inhibitors and progesterone-only pills may interfere with tubal functions.

3. Drugs impairing male fertility

The male of the human species is one of the species producing the lowest number of spermatozoa per gram of testicular tissue. His semen sample is often inferior to comparable mammalian samples.

3.1. Drugs affecting testicular functions

Some drugs, like antimitotics, have a direct global suppressive effect on testicular function. They impair both the endocrine and exocrine function of the testes.

3.1.1. Drugs impairing reproductive function

Many drugs in common use have reversible, or irreversible, effects on spermatogenesis. Some drugs exert a negative influence on sexual function and interest (libido).

Antibiotics, antibacterials, and some antimitotics, reversibly suppress spermatogenesis. Antimetabolites and radiotherapy, however, may produce irreversible damage to the gonads. It may be possible to reduce or avoid this damage by the administration of gonadal suppressants such as gonadotrophin-releasing hormone (GnRH) and testosterone (Pandiyan, 2000).

3.1.2. Antibiotics

Several antibiotics in common use have deleterious effects on sperm function or spermatogenesis. Ampicillins, cepholotin, dicloxacillin, penicillin G have all been found in animal studies to impair spermatogenesis, or inhibit the fertilizing capacity of the spermatozoa. Tylosin and lincomycin have been found to impair human sperm motility. Spiramycin was found to produce spermatogenic arrest. In human studies it has been found that tetracycline and erythromycin impair sperm motility. Neomycin impairs sperm concentration and motility. Gentamycin may produce premeiotic spermatogenic arrest (Hargreaves *et al.,* 1998).

3.1.3. Antibacterials

Quinolones may lead to impairment of spermatogenesis (von Rosentiel and Adam, 1994).

Sulfasalazine (Watkinson, 1986) and cotrimoxazole may impair sperm concentration, motility and morphology. Mesalazine, a common treatment for

ulcerative colitis, induces reversible infertility in men (Chermesh and Eliakim, 2004). Nitrofurantoin leads to reduction of sperm concentration by leading to premeiotic spermatogenic arrest. H2 receptor antagonists, including cimetidine and ranitidine, raise the serum prolactin concentrations and lead to loss of libido and erectile dysfunction. They may also cause gynaecomastia and reduction in sperm concentration.

3.1.4. Antimitotic agents, antimetabolites and other agents

The introduction of antimitotics, antimetabolites and chemotherapeutic agents in the management of malignant neoplasm has led to lasting remissions for many patients with acute lymphoblastic leukemia, Hodgkin's disease, gestational trophoblastic tumours and many other malignancies. This therapeutic success has raised concerns about persistent or delayed toxicities of cancer chemotherapy on long term survivors. Gonadal toxicity occurs in many men, women and children treated with antimitotic agents. (Schilsky *et al.,* 1980).

Colchicines and cyclophosphamide may lead to reversible or irreversible arrest of spermatogenesis and azoospermia. Corticosteroids may impair sperm concentration and motility. Hormones also have their disadvantages. Cyproterone acetate, danazol and finasteride may all lead to a reversible reduction of spermatogenesis. Halothane and local anaesthetics may impair sperm motility. Ketoconazole and spironolactone may all impair spermatogenesis, leading to oligozoospermia via their antiandrogenic activity. Methadone, phenothiazines, butyrophenone and niridazole may all depress spermatogenesis to cause low concentrations, and weak motility, of sperm. Quinine, its derivatives, and phenytoin, a commonly used anti-epileptic drug, may all decrease sperm motility. The calcium channel-blocker nifedipine may lead to fertilization failure (Pandiyan, 2000).

3.2. Drugs impairing spermatogenesis

The adult testis is the site of the highest ongoing mitotic cell division rate in the body. Most antimitotic, and several other drugs impair spermatogenesis. The effect is profound with alkylating agents. Abuse of anabolic steroids is a frequent cause of male infertility. Anabolic steroids impair spermatogenesis. can greatly affect the pituitary gonadal axis and induce a hypogonadal state with impaired spermatogenesis.

The following axioms apply to the gonadal toxicity of chemotherapeutic drugs in the male:

- Spermatogenesis is much more likely to be disrupted than is testosterone production, because the germinal epithelium of the testis is much more active and hence more sensitive to damage from cytotoxic drugs than the Leydig cells (Roeser *et al.,* 1978).

- The degree of damage to the germinal epithelium is influenced by the stage of sexual maturation of the testes. In general, the postpubertal testis, which is very active, appears to be more susceptible to damage than the prepubertal testis, which is relatively quiescent (Rivkees and Crawford, 1988).

- The magnitude of the effect on sperm production is both drug-specific and dose-dependent. Some drugs are more toxic than others (Roeser *et al.,* 1978).

- The newer anti-mitotics like cis-platin in the currently used dosage, though gonadotoxic, may over time lead to recovery of spermatogenesis (Port and Albrecht, 1997).

MECHANISM OF DRUG-INDUCED INFERTILITY — The mechanism of action of most chemotherapeutic agents consists of interference with obligatory cell processes, such as DNA synthesis, in the rapidly dividing cancer cells. However, all cells that undergo rapid division are susceptible to the toxic effects of chemotherapy.

Impact on the germinal epithelium — In the testis, the cells of the germinal epithelium have the highest mitotic and meiotic indices, and are thus most vulnerable to the toxic effects of chemotherapy (Roeser *et al.,* 1978). While sperm counts begin to decline within a few weeks of chemotherapy, it typically takes two to three months for azoospermia to occur, in keeping with the known kinetics of spermatogenesis. Because antineoplastic agents act on the sperm cells during cell division, they are most toxic to the rapidly proliferating type B spermatogonia, which can be reproduced from the germinal stem cell layer. However, the severity and duration of gonadal damage induced by cytotoxic agents correlates best with the number of stem cells (type A spermatogonia) that are destroyed. If the stem cells remain intact, one can expect spermatogenesis to show recovery approximately 12 weeks after treatment. Therefore, drugs that damage the stem cells are likely to cause permanent infertility (Damani *et al.,* 2002). The typical histological pattern on testicular biopsy of patients who have received cytotoxic agents is atrophic tubules containing Sertoli cells lining the lumen, a few scattered spermatogonia, spermatids, and peritubular fibrosis.

Impact on Leydig cells — Less commonly, chemotherapy can damage Leydig cells, the site of testosterone production within the testis (Howell *et al.,* 1999). However, Leydig cell dysfunction in this setting is typically subclinical, characterized by testosterone levels that are at the lower end of the normal range, in association with elevated LH levels. The clinical significance of this state of "compensated hypogonadism" is still unclear.

IMPACT OF CANCER ON GONADAL FUNCTION — It is well documented that certain malignancies may also be associated with pre-treatment abnormalities in testicular function, particularly Hodgkin's disease, and testicular, as well as extragonadal, germ cell tumours. The semen picture in these patients is abnormal even prior to starting treatment (Viviani *et al.,* 1991).

3.3. Drugs impairing sperm movement

Recreational drugs like marijuana, alcohol, nicotine and caffeine impair all semen parameters like volume, sperm movement and sperm concentration. In habitual marijuana users, the spermatozoa move too fast, too soon, and are consequently burnt out before they reach the oocyte. Marijuana interferes with arachidonylethanolmide (AEA) signalling which regulates sperm function (Schuel *et al.,* 2002). Alcohol in moderate to severe doses also impairs sexual function. As has been said by Shakespeare in Macbeth “lechery….it provokes and unprovokes; Alcohol provokes the desire but takes away the performance.”.

Smoking (nicotine) in men may lead to oligozoospermia, asthenozoospermia, or teratozoospermia. Smoking in men is associated with lower semen quality. Tobacco use in any form is associated with several reproductive disorders, and in men it is an important risk factor for erectile dysfunction.

3.4. Drugs affecting fertilizing capacity of the spermatozoa

Spermatozoa are specialised cells with their only function being to find, fuse and transfer their genetic material to the egg.

Fertilisation is a matter of life or death. Gametes that participate successfully in fertilisation grow as an embryo, and create another individual. Unsuccessful gametes die, and are lost. Gamete interaction involves ion channels. Sperm motility is directed by chemotaxis (Ral *et al.,* 1994), involves calcium ion movements, and is impaired by some anti hypertensive drugs.

Ion channels are essential elements in cell signalling and are fundamental to sperm physiology.

Nifedipine, a calcium channel blocker, interferes with the fertilising capacity of the spermatozoa and causes infertility.

3.5. Drugs impairing sexual function and fertility

Many medicines are suspected to interfere with sexual function, and about 25 per cent of cases of erectile dysfunction seen in clinics result from the side effects of medicines.

However, it is relatively difficult to determine whether a particular medicine can cause impotence or disrupt sexual function in other ways, for two reasons. First, many diseases themselves often affect sexual function, so it can be difficult to establish if the dysfunction is a result of the disease or the medicine used to treat it. 90% of severely depressed men report sexual dysfunction. Second, side effects that involve sexual issues are frequently not reported to doctors due to embarrassment. So, the actual rate of sexual dysfunction caused by medicines might be higher than reported.

4. Mechanism of sexual function

The mechanism of sexual function is not fully understood. It involves a complex co-ordination of hormones, chemical messengers in the brain (neurotransmitters such as dopamine and serotonin) and the actual sexual organs themselves. However, in general, the neurotransmitter dopamine increases sexual function, whereas the neurotransmitter serotonin inhibits sexual function. The hormone testosterone is important, as are the blood vessels involved in producing an erection. A medicine may therefore affect sexual function in several ways.

4.1. Libido or sex drive

Reproductive hormones influence sex drive, particularly testosterone, which is required for sexual arousal. Medicines that reduce testosterone levels are likely to reduce sex drive. Libido is also affected by general emotional and physical health. Therefore, medicines that affect any of these aspects, even indirectly by causing drowsiness, lethargy, weight gain or confusion, have the potential to reduce sex drive. Any elevation in prolactin level, due to lactotroph hyperplasia, a tumour, drug

intake or due to hypothyroidism, may reduce sex drive, with or without reduction in testosterone levels.

4.2. Arousal and erection

An erection occurs as a result of a co-ordination between nerves, hormones, blood vessels and psychological factors. There are therefore many areas where this can go wrong. Impotence (inability to get or sustain an erection) is not just caused by medicines that have a physical effect on the blood vessels in the penis, but also those acting on the brain, interfering with hormone levels (particularly testosterone), or affecting the transmission of nerve messages.

4.3. Ejaculation

This occurs after stimulation of alpha-receptors in the prostate gland and seminal vesicles. Medicines that block alpha-receptors can therefore prevent or delay ejaculation. Various chemicals in the brain are also involved in orgasm and ejaculation, and medicines that affect these chemicals can also cause ejaculatory disturbances.

4.4. Medicines that may affect sexual function

Antidepressants, particularly SSRIs (selective serotonin reuptake inhibitors) such as fluoxetine, are the medicines most frequently implicated in causing sexual dysfunction. This is because they work by altering levels of chemicals in the brain. In particular, SSRIs increase serotonin levels, which inhibit sexual function. Blood pressure lowering (antihypertensive) medicines is the other key culprits, most probably causing sexual problems by their effect on the smooth muscle and blood vessels in the penis.

Drugs like alpha methyl dopa and reserpine deplete the central dopamine stores and lead to hyperprolactinaemia and erectile dysfunction.

Drugs like phenothiazines (chlorpromazine), butyrophenones (haloperidol), and benzamides (metoclopromide, sulpiride and domperinone) block dopamine receptors. Blockage of endogenous dopamine leads to release of lactotrophs from the inhibitory control, resulting in hyperprolactinaemia and erectile dysfunction.

Table 14. Drugs affecting the spermiogram

Drugs or group of drugs	Effect of treatment
Antibiotics gentamycin, neomycin, penicillin G, cephalotin, ampicillin, spiramycin ***Antibacterials*** nitrofurantoin, sulfasalazine, cotrimoxazole ***Testosterone and its esters*** Injected testosterone,GnRH analogues,	Reversible suppression of spermatogenesis
Antimitotics/Antimetabolites cyclophosphomide, colchicines	Irreversible arrest of spermatogenesis and azoospermia
Antibiotics tetracycline, neomycin, erythromycin, lincomycin, tylosin, dicloxacillin ***Antibacterials*** sulphasalazine, cotrimoxazole, quinolones ***Anti-epileptics*** phenytoin ***Antimalarial*** quinine	Reversible impairment of sperm motility
Calcium channel blockers like nifedipine	Fertilization failure

Table 15. Drugs impairing sexual function

Drugs or group of drugs	Effect of treatment
H2 receptor antagonists' cimetidine, ranitidine.	Raise prolactin concentrations and lead to loss of libido and erectile dysfunction
Antipsychotic drugs Phenothiazine, antidepressants, a blockers	Raise prolactin concentrations and lead to loss of libido, erectile and ejaculatory dysfunction
Antihypertensives clonidine, guanethidine, hydralazine, methyl dopa, prazosin, beta blockers, thiazide diuretics,metoclopromide	Cause erectile dysfunction
Anticonvulsants spironolactone, finasteride, ketoconazole	Cause erectile dysfunction

5. Assessment of drug-induced impairment of fertility

A detailed history of chronic or short-term drug intake is important. While several drugs have been proved to produce impairment of fertility, careful documentation would help in bringing to light the impairment produced by several other drugs.

Compromise of ovarian function can be assessed by estimation of serum gonadotrophins, particularly FSH. Raised FSH and LH with very low oestradiol levels indicate compromised ovarian function. FSH is a fairly good indicator of ovarian reserve. In a cycling woman, FSH levels above 15miu/ml on day2/3 of menstruation indicate poor ovarian reserve and a decreased probability of pregnancy in future. An elevated prolactin level indicates the presence of prolactinoma, hyperplasia, or is suggestive of drug-induced elevation of prolactin levels.

Ultrasound monitoring of follicular growth would help in assessing follicular dynamics, and follicular growth impairment.

An impairment of male fertility can be assessed by a detailed semen analysis, serum FSH estimation and in some cases, serum LH and testosterone estimation. In men with impaired sexual function estimation of serum prolactin and testosterone would be of value.

DIFFICULTIES IN ASSESSING FERTILITY IMPAIRING EFFECTS OF DRUGS - Pregnancy is the gold standard for assessing the fertility of a couple. But fortunately, or unfortunately, drug assessment involves the fertility of two independent individuals. Though it takes two people to make a baby, the fertility impairing effect could be on any one member of the couple. But an impaired fertility of one partner could be compensated by normal fertility, or enhanced fertility, of the other partner. Though sexual side effects are fairly straightforward and patient perceived, fertility is far more complex and requires far more complex studies. Semen analysis, which remains the gold standard for assessing a man's fertility, is notoriously variable and remains unreliable as a single test for assessing man's fertility. Time to pregnancy, though a good biological indicator, depends on the fertility of the partner too. Therefore, assessment of fertility impairment by drugs is far more difficult, complex and requires a high index of suspicion, detailed history and fairly accurate long-term study of the exposed population.

5.1. Prevention of drug-induced impairment of fertility

The most important step in the management of drug-induced infertility is the awareness of fertility impairing nature of several drugs.

Like X-rays, drugs should be used sparingly in the reproductive age group; drugs with known reproductive toxicity should be replaced by less toxic equivalents.

If it is essential to use a drug that is a reproductive toxicant, it should be used for the minimum possible time at the least effective dose.

When chronic administration of a drug is essential, a periodic drug-free interval may help in the recovery of the gonads and reproductive function.

Use of gonadal suppressants, like GnRH agonist analogues and testosterone, for the duration of chemotherapy may help in reducing the gonadal toxicity of anti-mitotics (Pandiyan, 2000).

5.2. Preservation of fertility

Fertility of most men on chemotherapy is restored after cessation of therapy. However, where there is a risk of irreversible gonadal damage, fertility can be preserved by the following methods.

- Using gonadal suppressants as mentioned earlier.
- Cryopreservation of semen- all men in the reproductive age group undergoing gonadotoxic chemotherapy should be offered the opportunity to have their semen cryopreserved.
- Cryopreservation of testicular tissue (Pandiyan and Jequier, 1996). In young prepubertal boys undergoing chemotherapy, cryopreservation of testicular tissue, and later auto transplantation, may help in restoring/preserving fertility.

Fertility in women on chemotherapy may be preserved by:

(1) The use of gonadal suppressants.

(2) Ovarian hyperstimulation, oocyte collection and cryopreservation of oocytes, if appropriate.

(3) Ovarian hyperstimulation, oocyte collection, *in vitro* fertilisation and embryo freezing when appropriate.

(4) Cryopreservation of ovarian tissue and auto transplantation later.

ENVIRONMENTAL INFLUENCES ON FERTILITY - Human reproductive function has been observed to be sensitive to changes in the physical, psychosocial and chemical environment. Environmental toxicants are ubiquitous. Almost all aspects of the human environment are polluted. The atmospheric air, soil, water and consequently the food chain, are polluted. Noise pollution is a harsh reality in many urban conglomerates. Nobody is immune to the harmful effects of pollution. Even the so-called organic foods are grown in contaminated soil with contaminated water in contaminated air and hence are likely to have toxicants and may lead to impairment of life as well as reproductive function. Fish have been documented to carry dangerous levels of toxicants, which are dangerous to life and reproduction. Besides, global warming is a harsh reality and there is certainly danger to life and reproduction on earth.

The world is a global village. Epidemics have a tendency to become pandemics. Physical changes in one area have a telling global effect. 'The chaos theory', of Edward Lorenz has become a stunning reality. The 'butterfly effect' is there for all to see. An undersea earthquake near Indonesia, leads to a tsunami in several regions of Asia, even reaching the shores of Africa. The chaos theory has had a lasting effect on science and the environment. Aspects of chaos are seen everywhere around the world, from the currents of the ocean to the flow of blood in fractal blood vessels. Global trade, global travel, migratory birds, animals and fishes carry the infection to every corner of the world.

6. Conclusions

Every drug under the sun, including the sun (when used in phototherapy), has side effects; any drug, which is effective, is likely to have side effects. Meticulous documentation of drugs administered, high index of suspicion of side effects, having an open mind about every possible side effect, sometimes asking leading questions are all methods that may reveal impairment of fertility by commonly used drugs. Every drug should be considered suspect until proved otherwise. All couples with infertility should have their detailed drug history taken. This may sometimes include even *in utero* exposure to drugs and other toxic pollutants.

Many medical disorders and many drugs in common use have been known to cause reproductive impairment. It is often difficult to decide which causes the impairment, the drug, or the disease, or both.

Several commonly used drugs have been documented to impair fertility in men and women. This side effect of the drug depends on the dose of the drug, the duration of administration, and the age of the patient. Most drugs cause only a reversible impairment of reproductive function. However, some drugs, like antimitotics, even when used in the treatment of connective tissue disorders, may lead to irreversible impairment of fertility.

Several environmental pollutants have been implicated in the impairment of fertility: particularly male fertility. Men are supposed to be exposed to a sea of oestrogen-like substances; commonly used pesticides like DDT are oestrogen mimics.

The assessment of fertility impairment by drugs is often difficult. The absence of physical symptoms, the complexity of the laboratory tests, the ignorance of the practitioners and the embarrassment of the couple to discuss fertility and sexual issues may compound the problem.

Drug-induced infertility is largely preventable or manageable, by gonadal suppressants, fertility preservation by semen freezing or testicular tissue freezing, by oocyte or embryo freezing, or by ovarian tissue freezing.

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IMPACT OF AIR POLLUTION ON REPRODUCTIVE HEALTH IN NORTHERN BOHEMIA

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Summary

The effect of environmental pollution on reproductive outcomes has been studied in the context of an inter-disciplinary research program analyzing the impact of air pollution on human health in the Czech Republic.

Semen quality was evaluated in young men living in the Teplice District of Northern Bohemia where they are exposed to episodes of high air pollution during the winter months. This exposure was associated with decreased sperm morphology and motility, and increased DNA fragmentation and sperm aneuploidy. Sperm

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 207–224.

concentrations and total sperm counts in these men were not associated with exposure to air pollution. In a follow up study, conducted after measures had been taken to reduce the air pollution, associations of exposure with sperm morphology and motility were no longer apparent. However, DNA fragmentation consistently showed significant positive associations with exposure to higher air pollution (SO_2, PM_{10}). These findings suggest that exposure to episodic air pollution may result in damage to sperm DNA that could contribute to adverse effects on male fertility and male-mediated adverse pregnancy outcomes.

Studies of pregnant women and their infants suggest an impact of PM_{10} and carcinogenic polycyclic aromatic hydrocarbons on pregnancy outcome as evidenced by increased intrauterine growth retardation and lower birth weight. These data suggest that exposure to air pollutants during very early pregnancy (the time around conception) may adversely affect foetal growth. Pregnancy outcome results suggest that pregnant women and foetuses represent a sensitive sub-population, and that air pollution may be a significant risk factor.

Taken together, these studies provide evidence that air pollution may be detrimental to reproductive health.

1. Introduction

Regional air pollution in the Czech Republic increased dramatically with the advent of industrialization in the 1950s concurrent with increased burning of brown coal, with high sulphur content, for both home heating and industry. For example, sulphur dioxide (SO_2) emissions in Czechoslovakia amounted to 0.9 million tons in the 1950s and increased to 3.5 million tons by 1985 (Bencko, 1991). This increase of emissions was particularly pronounced in the mountainous region of Northern Bohemia where coal is extensively harvested from mammoth open-pit mines, and is used to heat homes and generate power for local industry (e.g. glass production, chemical manufacturing, and petrochemical industries). As a consequence of the environmental pollution in the mining districts of Northern Bohemia, consisting of high levels of sulphur dioxide (SO_2), nitrogen oxides (NO_x) and polycyclic aromatic hydrocarbons (PAHs) this region became recognized as one the most polluted in all Europe during the late 1980s (Moldan and Schnor, 1992; Benes *et al.,* 2001). Early warning signs of the impact of this pollution on the natural environment were evident in the 1960s with extensive deforestation, presumably due to the acid rain resulting from the elevated SO_2 levels. During the 1970s the first health

consequences of environmental pollution in Czechoslovakia were identified (Sram *et al.,* 2001).

In November 1990, the Czech government instituted a program to examine pollution in the mining districts of Northern Bohemia by selecting one mining district, Teplice, and establishing the research program "Impact of Air Pollution on Human Health (Teplice Program)" (Sram *et al.,* 1996). The district of Prachatice in Southern Bohemia was selected as a control district because it was considerably less polluted. The hypothesis in the Teplice Program has been that air pollution in the Teplice district adversely affects the health of the population. The principal objective of this program was to assess human exposure to toxic air pollutants, and to relate ambient concentrations of pollutants to health risks. The program was originally composed of 25 different projects. The Teplice Program (1991-1999). with the help of U.S. EPA and CEC (DG XII, PHARE II), monitored air pollution in both districts and implemented studies to evaluate its impact on human health. In the early 1990s these two districts were the only regions in the Czech Republic in which organic and inorganic air pollutants were thoroughly monitored. The Teplice Program included studies of a number of health outcomes, including respiratory and neurological effects in children, biomonitoring of mutagens in adults, pregnancy outcome, and reproductive health in young men. We review here the results of the projects on pregnancy outcome and on reproductive outcomes in young men from exposure to environmental mutagens.

2. Human semen quality and air pollution

The study of male reproductive health in the Czech Republic resulted from community concern about potential health effects of air pollution. Reproductive health studies were prompted by reports that rates of conception and incidence of congenital anomalies were affected by seasonal increases in air pollution (Sram *et al.,* 1990). To examine the potential relationship between the season of elevated air pollution and male reproductive health, we surveyed young men and evaluated their semen quality (Selevan *et al.,* 2000).

In the first study, conducted during 1993-1994, young men (age 18) living in the Teplice District, the highly industrialized district with seasonally elevated levels of air pollution, were compared to those from Prachatice, the rural district with relatively clean air. Surveys were scheduled for either late winter, after the season of higher air pollution, or at the end of summer when pollution was low. Participation included a physical examination, donation of a semen sample, and completion of a

questionnaire on health, personal habits and exposure to solvents and metals through work or hobby. Analysis of questionnaire data from 408 volunteers showed that the men from Teplice and Prachatice were similar in physical characteristics, personal habits and work or hobby-related exposures. Sixty-six percent (272) of these men donated a single semen sample for routine semen analysis (semen volume, sperm concentration, motility and morphology evaluated according to WHO, 1992 guidelines), computer-aided sperms motion analysis (Schrader *et al.,* 1992) and evaluation of sperm chromatin structure (DNA fragmentation) using the sperm chromatin structure analysis (SCSA) assay (Evenson *et al.,* 1994). Sample volume was measured in a 15 ml graduated centrifuge tube and an aliquot removed to determine sperm concentration by haemocytometer (World Health Organization 1992). For motility analysis, aliquots of semen were loaded into 20 μm-deep chambers, mounted on a heated (37^{o}C) microscope stage and video-taped (Selevan *et al.,* 2000). After all cycles were complete, videotapes were analyzed for motility and motion characteristics. The percentage of morphologically normal sperm was determined by examining 300 sperm per sample at 1000x magnification under oil immersion and classifying them according to strict criteria as described by the World Health Organization (1992). Frozen straws were shipped to South Dakota State University for analysis of sperm chromatin structure. Air pollution data (SO_2, NOx, PM_{10} and PAH) for the 90 days preceding sample collection approximately covers the process of spermatogenesis, epididymal transit and abstinence, and thus were used to estimate ambient exposure during this relevant time period.

Monitoring confirmed that levels of these air pollutants were considerably higher in Teplice than in Prachatice, and were higher in the winter than during the rest of the year in both districts (Figure 14). Sperm concentrations and total sperm counts in these men, although unaffected by exposure to air pollution, were at the low end of the ranges reported worldwide (Table 16). Indeed, more men than expected fell below the WHO reference value (1999) for semen volume (54% below 2.0 ml), sperm concentration (21% below 20 million/ml), and total sperm per sample (28% below 40 million sperm/sample). Short abstinence intervals in young men could account for these observations. However, even after omitting samples from men reporting less than two days sexual abstinence, the respective percentages are still relatively high: 51% below 2.0 ml, 19% below 20 million sperm/ml, and 21% below 40 million sperm per sample. Published data suggest that exposure to environmental pollution may contribute to a decline in sperm counts worldwide (reviewed by Jouannet *et al.,* 2001). However, our results do not support a relationship between either district of residence or exposure to air pollution and decreased semen or sperm production. Thus, although we do not know why young Czech men would have relatively a low sperm counts, this study suggests that this observation is not likely to be related to air pollution.

Table 16. Semen outcomes: summary and by district (Selevan *et al.,* 2000)

	Summary				Prachatice				Teplice			
Outcome	No.	Mean ± SD	Median	Range	No.	Mean ± SD	Median	Range	No.	Mean ± SD	Median	Range
Production of Viable Sperm												
Semen Volume (ml)	272	1.96 ± 1.06	1.8	0.5-6.0	118	2.09 ± 1.09	2	0.5-6.0	154	1.86 ± 1.03	1.7	0.5-5.5
Concentration (millions/ml)	272	61.2 ± 60.9	44	0-456	118	60.6 ± 66.3	39	0-456	154	61.7 ± 56.6	49.5	0-421
Total Count (millions/sample)	272	113.3 ± 119.2	81.5	0-780	118	119.3 ± 137	79	0-780	154	108.6 ± 103.7	82.1	0-624
% Motile*	256	33.6 ± 17.2	32.9	0-84	113	36.1 ± 17.9	36	0-75	143	31.6 ± 16.3	31.1	0-84
Total Motile (in millions)	256	44.2 ± 68.4	24.3	0-579.7	113	52.5 ± 82.5	27.6	0-579.7	143	37.5 ± 54.2	22.5	0-398.1
Total Progressive (in millions)[a,b]	228	33.3 ± 45.2	19.8	0.6-354.8	105	38.6 ± 54.2	22.2	0.6-354.8	123	28.9 ± 35.4	18	0.6-261.7
Sperm Structure												
% Normal Morphology*	262	17.8 ± 8	16.7	1-53.5	111	19.3 ± 8.6	17.7	1.0-53.5	151	16.6 ± 7.3	16	1.0-36.3
% Morphologically Normal Heads*	262	36.5 ± 10.1	35.5	10.7-76.0	111	39.3 ± 11	39	10.7-76.0	151	34.4 ± 8.7	33.7	15.0-60.7
SCSA Comp αt	266	20.2 ± 14	15.9	2.0-81.0	116	19.8 ± 12.1	15.9	2.7-57.6	150	20.5 ± 15.4	15.8	2.0-81.0
Quality of Sperm Motion - CASA[b]												
Straight Line Velocity (VSL)	228	44.3 ± 9.6	45	20.1-72.0	105	44.1 ± 9.6	45.2	21.5-65.7	123	44.5 ± 9.6	44	20.1-72.0
Curvilinear Velocity (VCL)	228	91.8 ± 20.8	90.9	48.6-139.3	105	93 ± 21.9	91.9	51.7-139.3	123	90.7 ± 19.9	90.1	48.6-132.3
Linearity	228	48.6 ± 8	49	28.0-69.0	105	48 ± 8.5	48	31.0-68.0	123	49.2 ± 7.5	49	28.0-69.0

*[a] Total Progressive = Total motile x % sperm with VSL ≥ 25 μ/sec. [b] Only for samples with at least 25 sperm tracks; VSL and VCL are in μ/sec. * Different by District, $p < 0.05$ by Wilcoxon test*

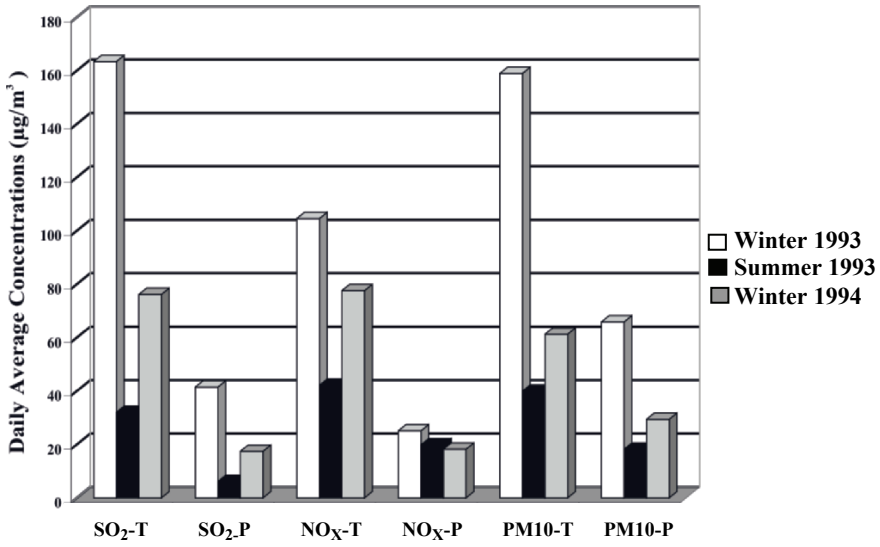

Figure 14. Air pollution data for Study I. Comparisons of levels for the 90 days prior to the survey for each collection period in Teplice (T) and Prachatice (P).

In all samples, the average percentage of motile sperm was 33.6% and the mean for Prachatice donors was slightly, but significantly, higher than that for Teplice donors. These mean values fall below the WHO reference value (1999) for percentage of motile sperm which is > 50%. Percent motile was also different by exposure category. Computer-assisted semen analysis, CASA, was used to evaluate the quality of sperm motion, and these results, while somewhat difficult to interpret, did not demonstrate any consistent negative associations between the quality of sperm motion and periods of high air pollution. The mean percentage of normal sperm for all samples was 17.8% (scored according to WHO 1992). The most recent WHO guidance (1999) does not specify a reference value for this measurement although multicenter population-based studies are underway to derive one using standardized "strict" criteria (as used in this study) for scoring each cell. Nevertheless, the guidance notes that as sperm morphology falls below 15% normal forms (using strict criteria for scoring sperm as "normal"), the fertilization rate *in vitro* decreases. Significant negative relationships were observed between district of residence and/or exposure to periods of medium (winter 1994 in Teplice) or high (winter 1993 in Teplice) air pollution and the percentage of sperm with overall normal morphology (considering head, midpiece and tail) as well as the percentage with normal head morphology. The significant association between exposure and poor sperm morphology was the most consistent finding in this report. The component(s) of air pollution responsible for such an effect or indeed whether the effect could be due to indirect factors (such as general stress) as opposed to any specific component of air

pollution remain to be explored. The SCSA was included in this study in order to provide a measure of the genetic integrity of sperm. Analysis of the SCSA data focused on the variable called DNA Fragmentation Index (DFI), the percentage of sperm with abnormal chromatin (i.e., demonstrating increased susceptibility to DNA denaturation in situ). DFI was significantly higher in samples obtained after the period of highest air pollution (winter 1993).

These results suggest that adolescent men exposed periodically to high air pollution are not more likely to have lower sperm counts. However, in this cohort, exposure to high levels of air pollution was associated with decreased sperm quality (morphology, motility) and increased DNA fragmentation. These results provided novel evidence that air pollution may be detrimental to male reproductive health.

3. Episodic air pollution and sperm DNA damage

The results of the first study, where each man provided only one sample, prompted a follow up study with a longitudinal design (Rubes *et al.,* 2005). This design with repeated measures allows each man to serve as his own control. Initiated in 1995, this longitudinal study was designed to overcome some of the limitations of the preliminary study.

Thirty-six men from the Teplice District, recruited from among the volunteers participating in the first study, were surveyed on 7 occasions over a 2-year period, again concentrating on late summer when pollution was low (September 1995, 1996 and 1997) and winter when pollution was high. Four samples were obtained during the winter (January, February and March 1996 and February 1997) when pollution was higher. A structured questionnaire, similar to that used in the first study, was administered to obtain information about each participant's reproductive and general health, including recent fever and use of medications and vitamins, as well as life-style factors and other potential exposures that could impact semen quality, including: smoking; alcohol and caffeine consumption; type of underwear; exposure to solvents, pesticides and metals through work or hobby. Semen outcomes, obtained as in the first study (Selevan *et al.,* 2000) were analyzed for changes associated with high levels of air pollution (SO_2, NO_x, PM_{10} or PAH) using a mixed model regression analysis for repeated measures, and checking for effect modifiers and potential confounders. As expected, air pollution levels, expressed as means for the 90 days preceding semen sampling, were higher in winter than in summer. However, exposures were lower during 1995-7 than during the previous study (Figure 15). Nevertheless, 90 day mean values approached or exceeded the upper

levels for annual US air quality standards for SO_2 (80 μg/m^3/day), NO_x (100 μg/m^3/day) and PM_{10} (50 μg/m^3/day). Furthermore, individual daily values exceeded the US 24 h limit (365 μg/m^3/day for SO_2 and 150 μg/m^3/day for PM_{10}) >1 day per year and were therefore out of compliance with US air quality standards.

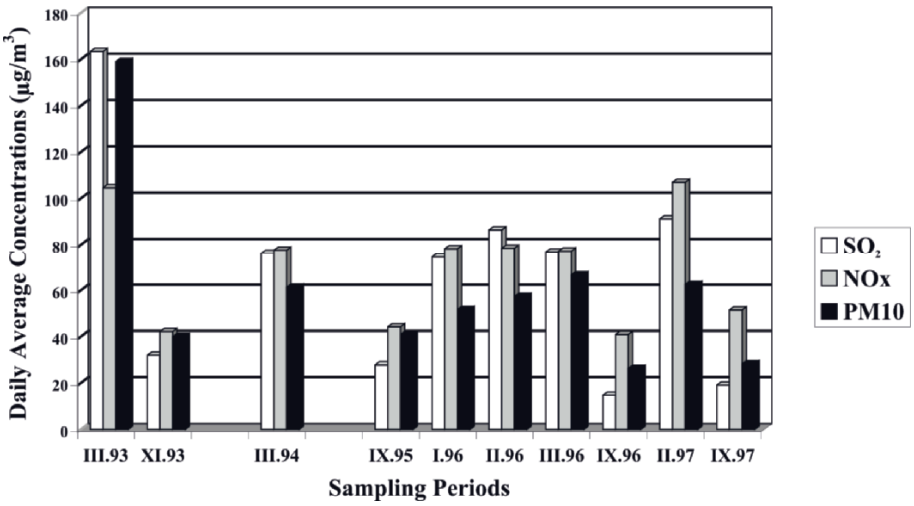

Figure 15. Air pollution data in Teplice – Study I versus Study II. Pollutant levels in the 90 days preceding the sampling periods.

Descriptive statistics for semen data (means and 95 percent CI) are given in Table 17. Shading of the columns shows the time periods when air pollution levels were higher (winter - sampling periods 2, 3, 4 and 6) versus the reference sample period (1, 5 and 7). No significant associations were found between exposure and sperm concentration, percent motile sperm, or percent normal sperm heads. Similarly, no significant associations were found between exposure and any of the three selected CASA measures. In contrast, the percent sperm with abnormal chromatin structure increased with exposure. Specifically, DNA Fragmentation Index (DFI), obtained using SCSA, showed significant ($p < 0.05$) positive associations with air pollution categorized as high vs low ($\beta = 0.19$, 95% CI: 0.02, 0.36), and with SO_2 levels ($\beta = 0.026$, CI: 0.001, 0.053). Correlations between DFI and either PM_{10} or PAH were of borderline significance ($p < 0.066$ and $p < 0.059$, respectively).

Table 17. Descriptive statistics for semen outcomes obtained by repeated sampling of 36 men 7 times over two years[1] (Rubes *et al.,* 2005) .)

Semen Endpoints	Sample 1 September 1995 LOW	Sample 2 January 1996 HIGH	Sample 3 February 1996 HIGH	Sample 4 March 1996 HIGH	Sample 5 September 1996 LOW	Sample 6 February 1997 HIGH	Sample 7 September 1997 LOW
Sperm Count (millions)	293.5 (223.0-364.0)	253.7 (167.4-340.0)	270.4 (181.2-359.6)	300.5 (211.8–389.1)	278.1 (197.9-358.2)	234.2 (188.1-280.3)	262.2 (178.0-346.3)
Sperm Concentration (million / ml)	98.6 (74.9-122.4)	78.5 (57.1-99.9)	79.9 (60.9-99.0)	103.1 (76.6-129.6)	92.1 (66.3-117.9)	81.6 (67.9-95.4)	103.6 (65.4-141.7)
Semen Volume (ml)	3.3 (2.7-3.9)	3.1 (2.4-3.8)	3.4 (2.7-4.1)	3.2 (2.5-3.9)	3.3 (2.8-3.8)	3.0 (2.5-3.6)	2.8 (2.4-3.1)
Motile Sperm (%)	58.5 (52.0-65.0)	55.0 (47.2-62.9)	59.1 (52.0-66.3)	66.3 (61.8-70.7)	62.7 (55.7-69.8)	68.3 (64.4-72.3)	56.2 (50.9-61.6)
Normal Sperm Head Morphology (%)	29.5 (25.8-33.1)	29.0 (26.6-31.3)	26.4 (23.7-29.1)	26.8 (23.6-29.9)	26.0 (23.4-28.6)	24.5 (23.1-26.0)	27.8 (26.4-29.2)
Normal Sperm Morphology (%)	17.5 (14.9,20.1)	14.8 (13.1,16.6)	15.8 (11.8-19.7)	12.7 (10.7,14.6)	11.3 (9.3-13.3)	8.4 (7.5-9.2)	7.9 (6.8-8.9)
Straight Line Velocity (µm/sec)	33.6 (31.4-35.7)	35.8 (31.8-39.9)	35.1 (32.9-37.2)	35.5 (33.7-37.3)	35.0 (33.0-37.1)	36.4 (34.9-38.0)	35.1 (33.2-37.1)
Curvilinear Velocity (µm/sec)	69.4 (66.6-72.1)	76.7 (68.4-84.9)	69.5 (65.0-74.0)	71.5 (68.2-74.7)	70.9 (66.6-75.2)	72.8 (69.1-76.5)	71.0 (67.0-75.0)
Linearity	50.9 (48.4-53.4)	51.1 (48.1-54.1)	53.8 (51.0-56.7)	52.2 (49.8-54.6)	52.2 (49.1-55.3)	52.4 (49.8-55.1)	51.6 (49.4-53.8)
SCSA-DFI (%)	15.1 (12.4-17.8)	20.3 (16.0-24.6)	15.8 (11.8-19.7)	17.4 (13.0-21.7)	13.5 (10.3-16.7)	15.4 (11.3-19.5)	12.2 (9.5-14.8)
Total Aneuploidy #/10,000 sperm	21.2 (16.0, 26.6)	24.2 (17.3-31.0)	22.6 (13.7-31.6)	20.1 (13.0-27.2)	21.3 (13.8-28.7)	18.5 (14.0-23.0)	24.0 (15.6-32.5)

[1] *Values are means (95% confidence interval) for each group of samples. Pollution is designated as "low" or "high" for the 3 months preceding each sample.*

[2] *Values for total aneuploidy represent the sum of total disomy and total diploidy and are based on 15 men who contributed 7 samples each.*

The objective of this study was to test whether exposure to intermittent high levels of air pollution was associated with decrements in semen quality. Consistent with the earlier one (Selevan *et al.,* 2000), this study found a significant association between exposure to air pollution and the percentage of sperm with fragmented DNA (SCSA–%DFI) thereby increasing the weight of evidence that exposure to high levels of air pollution may have damaging effects on sperm DNA. It is

biologically plausible that reactive metabolites of PAH might reach the testes and react with sperm DNA to form adducts. For example, other studies in the Teplice Program found that PAH inform the PM_{10} fraction can enter the body and form DNA adducts in at least two tissues, blood and placenta (Binkova *et al.,* 1995; Topinka *et al.,* 1997). Although DNA adducts in most germ cell stages should be repairable, DNA repair is not possible in the condensed spermatids and epididymal sperm in which protamine has replaced somatic histones (reviewed by Baarends *et al.,* 2001). Therefore, it is expected that toxicant-induced DNA damage in this repair-deficient period of late spermiogenesis and epididymal sperm maturation, or about the last 10 days before ejaculation, would not be repaired and could therefore be manifest as increased SCSA–%DFI. Mean baseline values for SCSA–%DFI for this cohort were within a range considered normal (12–15%) and increased to 15–20% after exposure. Although the increase was statistically significant, these levels are still considered indicative of good fertility potential (Larson *et al.,* 2000). Based on clinical studies, however, when SCSA–%DFI approaches and exceeds 30% the risk for infertility and spontaneous miscarriage is considerable, even in men with otherwise good semen quality (Evenson *et al.,* 1999; Larson *et al.,* 2000; Spano *et al.,* 2000; Zini, 2002; Larson-Cook *et al.,* 2003; Virro *et al.,* 2004). Although the change in average SCSA–%DFI observed in this study may not have affected the fertility potential of these individual men, changes of this magnitude could impact fertility of men in the general population who have higher baseline SCSA– %DFI. Thus, when evaluating environmental risks to the general population, even modest increases in SCSA–%DFI may impact fertility in those men at the higher end of the distribution of SCSA–%DFI. Further study of the association between air pollution and SCSA-%DFI is needed in order to test the hypothesis that reactive metabolites of PAH may damage DNA.

In contrast to the positive association between air pollution and SCSA-%DFI, no significant associations were found with sperm morphology or motility (percentage motile sperm), as had been observed in the first study. This lack of consistency between studies may be related to the remedial actions by the Czech government that resulted in a decline in air pollution between 1993 and subsequent years (Pinto *et al.,* 1998; Benes *et al.,* 2001). With specific reference to the two semen studies described above, mean SO_2 levels for comparable 90 days intervals (late December to late March) were notably higher in 1993 (164.0 $\mu g/m^3$, Selevan *et al.,* 2000) compared with 1996 (78.5 $\mu g/m^3$). The same was true for PM_{10} where the comparable 1993 mean was 184.7 $\mu g/m^3$ (Selevan *et al.,* 2000) compared with 67.8 $\mu g/m^3$ for 1996. In a subsequent study conducted in 1998, another group of 50 eighteen-year old men were similarly examined (unpublished data). Comparison of

mean values for the first group of men sampled in spring 1993 to this group sampled in 1998 showed that sperm concentration and percentage of motile spermatozoa were significantly higher (60.1 vs 102.3 mil/ml and 32.5 vs. 62% motile, respectively) in the 1998 group. Taken together, the three studies provide evidence that exposure to high levels of air pollution is associated with decrements in semen quality, and that these appear to be reversible once the pollution is lowered.

4. Air pollution and sperm aneuploidy

Traditionally, the effects of environmental exposures on human germ cell chromosomes have been studied indirectly by using epidemiological investigations of adverse reproductive outcomes such as reduced fertility, spontaneous abortion, birth defects, and childhood cancer. However, a growing number of new molecular genetic techniques allow *direct* measurement of germ cell cytogenetic damage and a number of these technologies are proving useful in human studies of environmentally induced germ cell damage. Aneuploidy is one of the most serious and common chromosomal abnormalities affecting human embryos and offspring (reviewed by Hassold and Hunt, 2001). Aneuploidy is also one of the major categories of genetic defects that can be transmitted via sperm (Wyrobek *et al.,* 2000). From a public health perspective, it is important to know if environmental contaminants induce cytogenetic damage in human sperm.

We used fluorescence in situ hybridization (FISH) techniques to detect numerical chromosomal abnormalities in human sperm. Air-dried smears of sperm were decondensed and hybridized immediately using fluorescent chromosome-specific α-satellite or satellite III DNA probes for chromosomes X, Y and 8. This method allows distinction between diploid and disomic sperm nuclei, and meiosis I and meiosis II errors in sex-chromosomal aneuploidy and diploidy. Slides were randomized and 10ˆ000 sperm were scored per sample, using strict scoring criteria (Robbins *et al.,* 1995). The number of disomic sperm (exhibiting signals for XX8, YY8, XY8, X88 or Y88) per sample was recorded and the total number of disomic sperm per sample (per 10ˆ000 cells) was calculated. The same was done for the number of diploid sperm (exhibiting signals for XX88, YY88 or XY88). Finally total disomies and total diploidies were summed.

Subsets of men (N=32) from the first study (Selevan *et al.,* 2000) were evaluated for sperm aneuploidy (Robbins *et al.,* 1999). Specimens collected in the early Spring of 1993 in Teplice after 3 months of exposure to the highest air pollution (average SO_2 levels 196.9 $\mu g/m^3$, standard deviation 196.8) were compared to those collected in

the Fall of 1993 after the relatively cleaner summer months (average SO_2 levels 32.0 $\mu g/m^3$, standard deviation 13.6). The sex chromosomal aneuploidy, disomy YY, was found to be five-fold higher in sperm following periods of exposure to high air pollution compared to low exposure. All men in the study were healthy, 18 year old non-smokers. Adjusting for potential confounders (alcohol, caffeine intake, fever, laboratory variables) did not change the effect estimate IRR 5.25, 95% CI 2.5 – 11.0 (Poisson regression modelling).

The sperm aneuploidy assay was conducted also for subsets (n = 15) of men from the longitudinal study (Rubes *et al.,* 2002, 2005). The men were healthy upon examination on entry into the study and did not report drug use, or occupational exposure, and declared themselves as either non-smokers or light smokers. Seven semen specimens were provided by each of 15 healthy men over a 2 year period (1995 – 1997), as described above, and evaluated by the X-Y-8 multi-colour sperm FISH method. Descriptive statistics for aneupoidy data are given in Table 17. There was no significant effect of season (high or low pollution) on aneuploidy or diploidy for any of the chromosomes studied. Inconsistencies between studies could be due to differences in the exposures as discussed above. The sperm disomy findings for the Teplice non-smokers compared closely with those of a group of Californian non-smokers. The slides for both cohorts were prepared and scored in the same laboratory using the same probes and scoring criteria, but different scorers. Overall, there were no differences in the levels of sperm diploidies or Y disomy. The Czech non-smokers, however, were elevated in the levels of X and 8 disomies and XY sperm ($p = 0.02$). These differences may be related to the geographic and ethnic differences, pollution differences, or cultural differences between the two cohorts.

Several factors have been found to increase a male's risk of producing aneuploid sperm, including age (reviewed by Sloter *et al.,* 2004), treatment with aneugenic cancer chemotherapy (reviewed by Martin, 2003), and certain life style factors (Robbins *et al*., 1997; Rubes *et al.,* 1998; Harkonen *et al.,* 1999; Shi *et al.,* 2001). Numerous studies also describe a relationship between male infertility, poor semen quality, and increased levels of sperm disomy (reviewed by Tempest and Griffin, 2004). A few epidemiology studies have examined potential relationships between other types of environmental exposures and sperm aneuploidy. Specific studies on the effects of pesticide exposure on germ cell aneuploidy show conflicting results (Smith *et al.,* 2004).

Our findings suggest an effect of air pollution on human germ cell chromosomes that warrants further investigation.

5. The impact of polycyclic aromatic hydrocarbons and fine particles on pregnancy outcome

The principal aim of the Pregnancy Outcome project was to evaluate the possible impact of air pollution on intrauterine growth retardation (IUGR). IUGR is operationally defined as infants below the 10th percentile of birth weight for gestational age and gender. The study group includes all singleton full-term births of European origin over a 2-year period in the Teplice District. Information on reproductive history, health, and life style was obtained from maternal questionnaires. Using continuous monitoring data, mean concentrations of pollutants were calculated for each month of gestation and for each mother. Odds ratios (ORs) for IUGR for PM_{10} and PM 2.5 levels were generated using logistic regression for each month of gestation after adjustment for potential confounding factors (Dejmek *et al.,* 1999).

A significantly increased risk of giving birth to a child with IUGR was established for mothers who were exposed to PM_{10} levels >40 $\mu g/m^3$ or PM2.5 > 27 $\mu g/m^3$ during the first month of gestation. For each 10 $\mu g/m^3$ increase in PM_{10}, the AOR (Adjusted Odds Ratio) of IUGR was 1.25 (CI 1.08-1.56); a similar, but weaker association was also observed for PM2.5. No association of IUGR risk with particle levels was found in any later gestational month. No association with IUGR was observed for sulphur dioxide, nitrous oxides or ozone (Dejmek *et al.,* 1996). The influence of fine particles on foetal growth was later reanalyzed in a four-year data set (Dejmek *et al.,* 2000). The IUGR risk was 1.44 (CI 1.03-2.02) for medium levels (PM_{10} = 40 to <50 $\mu g/m^3$) and 2.14 (CI 1.42-3.23) for high levels of PM_{10} (PM_{10}= 50 or more $\mu g/m^3$) during the first month of gestation. Analyzing continuous air pollution data, a dose-effect relationship was again confirmed: for each 10 $\mu g/m^3$ increase of PM_{10} in the first gestational month, the AOR was 1.19 (CI 1.06-1.33).

Analyzing the same cohort, Dejmek *et al.* (2000) tested the association between carc-PAHs and IUGR. Analyzing the Teplice data, a highly significant increase of IUGR risk was found for exposures to carcinogenic PAHs (carc-PAHs, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,I]perylene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-c,d]pyrene) > 15 ng/m^3 – again exclusively during the first gestational month. The AORs for medium levels of carc-PAHs were 1.59 (CI 1.06-2.39) and for high levels 2.15 (CI 1.27-3.63). This relationship proved to be strongly dose-response related: per 10 ng/m^3 elevation of carc-PAHs level, the AOR was 1.22 (CI 1.07-1.39). In contrast to the previous negative effects of PM_{10} in Prachatice, the association between carc-PAHs and IUGR was close to that found in Teplice

(Dejmek *et al.,* 2000). Again, the only consistent carc-PAHs/IUGR association in Prachatice was observed in the first gestational month. AORs for medium levels of carc-PAHs in Prachatice were 1.63 (CI 0.87-3.06) and for high levels 2.39 (CI 1.01-5.65).

The molecular epidemiological studies suggest biological mechanisms for the effect of air pollution, especially carc-PAHs on birth outcomes. It has been shown that the levels of DNA adducts are positively related to risk of IUGR (Sram *et al.,* 1999), birth weight, birth length and health circumference (Perera *et al.,* 1999), and hypoxanthine-guanine phosphoribosyl-transferase locus (HPRT) mutation frequency in infants (Perera *et al.,* 2002). The genotoxic risk of air pollution may be further affected by genetic polymorphisms of genes affecting xenobiotic metabolism as well as DNA repair (Sram and Binkova, 2000).

These data suggest that the primary role in elevation of IUGR risk is due to exposure to these carcinogenic PAHs. This finding is consistent with the idea of a primary role for carc-PAHs in foetal growth modulation (Guyda, 1991; Zhang *et al.,* 1995).

6. Conclusions

The results of the studies in Northern Bohemia have important implications for reproductive risk assessment: First, they suggest that exposure to even short episodes of high levels of air pollution may have adverse effects on male and female reproductive function. Second, while such effects are likely reversible in a particular man, an affected sperm may transmit genetic damage to his conceptus, resulting in early pregnancy loss or other adverse developmental outcomes. Third, this program identified developmental changes in early stages of pregnancy associated with air pollutants as e.g. carc-PAHc

The Teplice Program results have become the basis for the Czech Government's decision to support changing Northern Bohemia's heating from coal to gas which, together with desulphurization of power plants in that area resulted in substantial decreases in concentrations of sulphur dioxide over a ten year period: concentrations in 1999 were approximately 15% of those in 1990. In addition, decreases of respirable particulate matter (PM_{10}, <10 μm) and carcinogenic polycyclic aromatic hydrocarbons (PAHs) were observed. Even with these measures to reduce air pollution, the concentrations of PM_{10} and PAHs decreased less than was originally expected after 1993. Therefore PM_{10} and PAHs remain a cause for concern.

Acknowledgements

This work was supported by grants VaV 340/1/1997 and VaV/340/02/00 from the Czech Ministry of the Environment and the Ministry of Agriculture (MZE 0002716201).

Disclaimer: This paper has been reviewed by the US EPA, National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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SECTION 4:

POLICY INSTRUMENTS AND ISSUES

PESTICIDES AS ENDOCRINE DISRUPTERS: *IDENTIFICATION OF HAZARDS FOR FEMALE REPRODUCTIVE FUNCTION*

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Summary

Several pesticides are potential endocrine disrupters, with mechanisms including estrogen receptor agonism (chlorinated insecticides), aromatase inhibition (triazoles), interference with pituitary-gonadal axis (chloro-S-triazines) or thyroid (ethylene bisdithiocarbammates). Therefore, female reproductive function may be regarded as a sensitive target for several pesticide groups. Toxicological studies indicate that such compounds may alter different steps of the reproductive cycle, including e.g., the development of the reproductive system as well as ovulation and implantation. Moreover, indirect effects on balance among hormones may be as important as direct actions on target receptors or tissues. However, several phases under endocrine control (e.g., parturition) are different between humans and rodents, thus, direct extrapolation of findings may be difficult on occasion. Consistent epidemiological findings indicate that occupational exposures to certain pesticides (phenoxy herbicides, triazines, glyphosate, thiocarbamates) are associated with miscarriage and reduced fecundity, also upon exposure of the male partner; concurrent factors may increase the risk, such as inadequate protective equipment and woman's age. As for the general population, the exposure to pesticide residues in vegetable foods appears under control in Europe. However, more data are needed on feed contamination leading to residues in foods of animal origin as well as on

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 227–248.

exposure through the living environment, e.g., from pest control activities. Moreover, there are no data to evaluate the health implications, if any, of such exposures. Thus, novel approaches are needed, including the development of appropriate biomarkers and biomonitoring programmes. Exposure to pesticide remains a potential concern for reproductive health; accordingly, current efforts of the European Commission toward sustainable use of pesticides, food safety and improved evaluation of environment and health relationships deserve support and implementation.

1. Introduction

The possible effects of environmental pollutants on female reproductive function have received till now comparatively smaller attention than those on male fertility or prenatal development. For instance, much debate and research has focussed on increasing trends of male reproductive alterations (from hypospadias to poor semen quality to seminomas) observed in several industrialized countries; such alterations have been linked in a "testicular dysgenesis syndrome" related to altered endocrine homeostasis in utero, for which a role of widespread exposure to xenobiotics has been suggested (reviewed by Sharpe, 2003). As regards female reproductive disorders, endometriosis is an example of a major gynecological entity for which there is an increasing interest in possible environmental risk factors (Rier, 2002; Yang *et al.,* 2000). Nevertheless, the adverse effects of diverse chemicals on female fertility and pregnancy maintenance have already been identified by a number of studies (Hruska *et al.,* 2000). Epidemiological evidence points to life style factors (cigarette smoke, high alcohol consumption, obesity) and intensive occupational exposures to, e.g., heat, radiation or solvents as risk factors for reproductive dysfunction in women, even though with variable strength (Eggert *et al.,* 2004; Hassan and Killick, 2004; Kumar, 2004). Moreover, increasing data on both humans and experimental species support the positive association between the body burden of persistent chlorinated pollutants (dioxins, DDT and metabolites, PCBs) and enhanced risk of gynecological problems including endometriosis (Rier, 2002;) and pregnancy loss (Gerhard *et al.,* 1999; Korrick *et al.,* 2001). Conflicting observations do exist: a careful study on Japanese patients with a history of recurrent miscarriage has not found an association with serum levels of PCBs, hexachlorobenzene or DDE (Sugiura-Ogasawara *et al.,* 2003). Thus, possible mechanisms related to different vulnerability deserve more attention. Nevertheless, the overall data support the public health concerns for Endocrine Disrupting Chemicals (EDC); EDC are a heterogeneous group of compounds including persistent pollutants, industrial chemicals and compounds used in agriculture and animal farming, all sharing the

ability to interfere with endocrine homeostasis (European Commission, 2004a). EDC are a somewhat different, and broader, topic as compared to more conventional reproductive toxicants due to a) the diversity of mechanisms by which EDC may act (Neubert, 1997) b) the multitude of endocrine-regulated targets that may be affected as well as the complexity of the interactions among the different systems (Harvey and Johnson 2002); c) the potential to induce long-term effects upon exposure during the early life stages (Mantovani, 2002); last but not least d) the potential widespread exposure of the general population, especially through the food chain (Guenther *et al.,* 2002; Schecter *et al.,* 2001).

Pesticides represent a significant portion of EDC, and to these one may add some similar compounds used as antiparasytic drugs in animal farming (Mantovani and Macrì, 2002). It is noticeable that EDC used as pesticides are pretty diverse concerning chemical structure and that they may elicit different mechanisms of endocrine disruption, including receptor interaction (e.g., vinclozolin), inhibition of steroid synthesis (e.g., triazoles), interfering with thyroid function (e.g., ethylene bisdithiocarbamates) and/or with pituitary axis (e.g., triazines) (Mantovani, 2002; Neubert, 1997). Thus, different critical targets and effects on reproductive function may occur.

As from the point of view of exposure, pesticides are rather unique among pollutants in that they are deliberately inserted into the environment in order to support crop production, and also animal farming, rather than being accidentally released. Thus, three different exposure scenarios may occur i) occupational, ii) environmental exposure, especially for communities living in areas of intensive agriculture (Curl *et al.,* 2002) and iii) dietary exposure of the general population through residues in foods of both vegetable and animal origin.

The present paper aims at reviewing the evidence on pesticides as possible risk factors for female reproduction, with special attention to hazards related to endocrine disruption, and also to identify needs for further research and potential public health concerns.

2. Toxicological studies

2.1. Identification of relevant mechanisms

Female reproductive function is a complex process involving hormonal balance and interplay, development and function of the different comparts of the ovary, uterine function, implantation and placentation (Harvey and Johnson, 2002). Although the

standardized *in vivo* regulatory reproductive toxicology studies provide the most useful tools to identify adverse effects resulting from endocrine disruption – including those on the female reproductive function – they are not specifically designed to analyse endocrine disrupting mechanisms (Mantovani, 2002). Further tests (*in vivo* or *in vitro*) or additional endpoints would be required to clarify the actions of the chemicals and the mechanisms operating (Baker, 2001). Accordingly, the Organisation for Economic Co-operation and Development has undertaken an international validation program for *in vivo* tests on endocrine disruption; validation has been completed for the rodent uterotrophic bioassay as a test to identify estrogen agonists or antagonists (Gelbke *et al.,* 2004).

Other examples included the following *in vitro* assays: i) MCF7 cell proliferation assay as well as yeast cells transfected with estrogen receptor alpha to identify estrogenic activity (Andersen *et al.,* 2002; Vinggaard *et al.,* 1999); ii) human placental microsomes, to identify interference with CYP19 fraction (aromatase, catalysing convestion of testosterone into estradiol 17-beta) (Andersen *et al.,* 2002; Vinggaard *et al.,* 2000); iii) luciferase expression assay for aryl hydrocarbon receptor (AhR-CALUX) in rat and human cell lines to detect "dioxin-like" toxicity (Long *et al.,* 2003). Several pesticides most widely used in Denmark were tested using these assays. The fungicide fenarimol (pirimydine derivative), was, somewhat unexpectedly, markedly positive in both assays for estrogenicity; two other fungicides, triadimephon (azole) and triadimenol (conazole) were significantly positive in the MCF7 assay whereas the chlorinated compound dicofol was significantly positive in transfected yeast cells, but only weakly in MCF7 (Vinggaard *et al.,* 1999). As expected from their known effects in different systems, the antifungals imazalil (imidazole), prochloraz and propioconazole (triazoles) were aromatase inhibitors *in vitro*; however, other compounds proved to be aromatase inhibitors as well, such as fenarimol, dicofol and another chlorinated insecticide, endosulfan (Andersen *et al.,* 2002; Vinggaard *et al.,* 2000). This latter was previously identified as an estrogenic compound *in vitro* (Soto *et al.,* 1995). In rat Leydig cell carcinoma and human adrenocorticocarcinoma cell lines, prochloraz and epoxyconazole confirmed to be aromatase inhibitors, whereas the chloro-S-triazine herbicide atrazine was a strong inducer, albeit in human cell line only (Heneweer *et al.,* 2004). As for AhR-agonism, a mechanism related to both endocrine and immune balance, for which little data exist on pesticides, the organophosphorus insecticide chlorpyrifos and the triazole prochloraz showed a significant, dose-dependent effect in both rat and human cell lines (Long *et al.,* 2003).

Till now, no individual *in vitro* assay is able to fully characterize the potential of a molecule for disrupting the endocrine homeostasis in the female organism. On the

other hand, the development of integrated batteries of *in vitro* assays may result in additional information, e.g., the potential for endocrine interference of the pyrimidine fungicide fenarimol (Vinggaard *et al.,* 1999; Vinggaard *et al.,* 2000). Nevertheless, findings *in vitro* cannot be automatically taken as predictive of effects in whole mammalian organisms. An example is endosulfan, an estrogen agonist *in vitro*, which failed to show estrogenic activity in ovariectomized mice, as shown by such standard endpoints as uterine weight and vaginal cornification (Hiremath and Kaliwal, 2003). The absence of estrogenic activity *in vivo* was confirmed in a further experiment evaluating the effects of a combined exposure with endosulfan and the pyrethroid deltamethrin in the offspring of rats treated during pregnancy and lactation (Presibella *et al.,* 2005).

2.2. Identification of hazards for female reproductive function elicited by different pesticides

Azole fungicides. Azole fungicides inhibit steroid biosynthesis in fungal organisms but also in mammals. They target the P450 enzyme pathway, in particular sterol 14-alpha-demethylase and aromatase; although triazoles and imidazoles (e.g., imazalil) appear to target mainly aromatase and 14-alpha-demethylase, respectively, in fact both enzymes may be considered common targets of azole compounds (Zarn *et al.,* 2003). Aromatase inhibitors typically induce female mediated parturition difficulties in laboratory rodents, resulting in dystocia and increased perinatal death. In particular, such effect is associated with the attenuation of the sharp decline in progesterone levels that physiologically occurs in rats and mice over the last few days of pregnancy; in fact, reduced progesterone production by ovarian corpora lutea is a trigger for the onset of labour in small rodents. Thus, the dystocias consistently observed in toxicological studies may be considered a species-specific effect (European Commission, 1999). This does not mean that aromatase inhibition does not deserve attention as a potential mechanism for endocrine disruption. Guinea pigs have been suggested as laboratory species more suitable to assess reproductive effects of aromatase inhibitors in humans, because of their greater similarities to humans concerning the endocrine control of pregnancy and parturition (European Commission, 1999). Prenatal aromatase inhibition impairs the negative feedback control of LH in sexually mature guinea pigs (Choate and Resko, 1994); however, there is a remarkable paucity of published reproductive toxicology studies in this species. Pesticides inhibiting steroid biosynthesis, such as the triazole cyproconazole, may also significantly increase early postimplantation loss in rats (Machera, 1995). A relationship with endocrine-mediated effects may not be ruled out, since delayed embryo implantation as well as impaired decidualization is

induced by drugs selectively interfering with steroidogenesis (Cummings *et al.,* 1997; Tamada *et al.,* 2003).

Chlorinated insecticides. Some chlorinated insecticides, such as methoxychlor, have an estrogenic activity *in vivo* (Kanno *et al.,* 2003). Methoxychlor induces premature, and abnormal, sexual maturation and alters ovarian cyclicity when given orally to peripubertal monkeys (Golub *et al.,* 2003) or to mice from fetal through to early postnatal phase (Masutomi *et al.,* 2003); thus a potential hazard for the maturation of reproductive function is identified. Other chlorinated compounds, may have different mechanisms. For instance, endosulfan may alter the estrogen-progesterone balance in mice at doses as low as 4 mg/kg b.w, possibly interfering with the pituitary-ovarian axis; effects include increase in the number of atretic follicles, disrupted estrous cycle and inhibited embryonic implantion (Hiremath and Kaliwal, 2002a; Hiremath and Kaliwal 2002b). Similar effects were also observed for dicofol in rats, suggesting an action on the endocrine balance analogous to endosulfan; however, dicofol appears to be a less potent disrupter, as markedly higher dose levels were required to affect reproductive function (Jadarmkunti and Kaliwal, 1999; Jadarmkunti and Kaliwal, 2001).

*Cholinesterase inhibitors***.** Organophosphorus insecticides and, to a lesser extent, carbamate insecticides are well known for their potential to induce acute neurotoxicity through the inhibition of brain acetylcholinesterase. A series of studies in mice indicated that the organophosphorus compounds dimethoate and monocrotophos and the carbamate carbofuran may reduce ovarian and uterine weight, number and size of healthy ovarian follicles and corpora lutea as well as the number of estrous cycles and the duration of fertile phases; exposure during early pregnancy may induce pre-implantation loss (Baligar and Kaliwal, 2002; Baligar and Kaliwal, 2003**;** Mahadevaswami and Kaliwal, 2002; Mahadevaswami and Kaliwal, 2003; Rao and Kaliwal, 2002). Findings might indicate either a direct effect on the ovary or an imbalance at any stage of the hypothalamo-pituitary-ovarian axis; however, they should be interpreted with caution, as general toxicity (e.g., reduced body weight) was evident in all studies and a contribution to reproductive effects cannot be ruled out. However, several recent *in vitro* studies indicate that some orgonophosphorus and carbamate insecticides (e.g. methiocarb, tolchlofos-methil) may act as agonists of estrogen receptors (ER) alpha and/or ERbeta; noticeably compounds such as methiocarb were predominantly agonists of ERbeta rather than of ERalpha (Grunfeld and Bonefeld-Jorgensen, 2004; Hofmeister and Bonefeld-Jorgensen, 2004; Kojima *et al.,* 2004). On the other hand not all cholinesterase inhibitors have to be considered to be potential endocrine disrupters; for instance, the organophosphate fenitrothion did not induce endocrine or

reproductive effects in rats exposed to ≤ 60 mg/kg feed in utero and from weaning to maturation, even though the treatment elicited a significant reduction of brain cholinesterase (Okahashi *et al.,* 2005).

Thiocarbammates. The dithiocarbammate fungicide thiram delays ovulation in rat, resulting in a reduced fertilizability of the released oocytes as well as reduced litter size (Stoker *et al.,* 2003). Since treatment was performed intraperitoneally, it is difficult to extrapolate such effect to a hazard following the expected ways of intake in humans (i.e., oral, inhalation, percutaneous); nevertheless, the findings may deserve further attention.

The ethylene bisdithiocarbammates (e.g., mancozeb) are fungicides which potentially interfere with thyroid function; their common metabolite, ethylene thiourea is of particular concern (Panganiban *et al.,* 2004). Mancozeb inhibits implantation in mice at dose levels ≥ 24 mg/kg b.w; the compound reduces also the relative weight of uterus and ovaries (Baligar *et al.,* 2001; Bindali and Kaliwal 2002). A negative feed-back mechanism on estrogen production or activity cannot be excluded. Moreover, exposure of rats to ethylene thiourea during organogenesis induces high rate of postimplantation loss, possibly related to a teratogenic effect and/or to maternal hypothyroidism (Houeto *et al.,* 1995).

Triazines. The herbicide atrazine reduces the production of pituitary hormones such as prolactin (prl) and luteinizing hormone (LH) in female rats, most likely by disrupting the hypothalamic control of pituitary function (Cooper *et al.,* 2000). As prl promotes progesterone secretion, which is essential for the initiation of pregnancy in rats, atrazine increases early postimplantation loss and full litter resorption in this species (Cummings *et al.,* 2000). Such effect is maternally mediated, and consistent with loss of LH support of the corpora lutea (Narotsky *et al.,* 2001, McMullin *et al.,* 2004). Moreover, atrazine may delay puberty and mammary gland development in female rats through either prenatal or lactational exposure (the latter possibly through altered transfer of growth factors or hormones) (Rayner *et al.,* 2004). However, atrazine-induced reproductive alterations are observed at high dose levels (≥ 100 mg/kg bw) and significant differences in sensitivity between rat strains do exist (Cummings *et al.,* 2000; Narotsky *et al.,* 2001); thus, health hazards may be unlikely at the expected human exposure levels (Gammon *et al.,* 2005).

3. Exposure to pesticides and reproductive problems in women

The assessment of potential adverse effects of pesticides exposure in women can be considered from several standpoints. In particular, the evidence of direct exposure through occupational use should be considered. Garcia (1998) provided a useful review of the papers published until the 1990s. Available studies deal mostly with overall exposure to pesticides, though a few papers try to associate adverse reproductive outcomes with a specific group of compounds.

A second aspect that might have a broader public health impact is the exposure of the general population through the diet and/or the environment. However, very few epidemiological data are available till now.

3.1. Is there an effect resulting from occupational exposure?

Manufacturing workers experience a direct exposure to pesticides; however, to our best knowledge, there are no published studies on the reproductive health of women employed in pesticide factories. Working on a farm or, indeed, living in a farm may themselves lead to pesticides exposure. Certain factors involved in agricultural work may, in fact, be a risk-factor for female fertility. Such factors may include use of pesticides as well as certain life style choices (alcohol consumption, smoking and passive smoke exposure, steady weight gain) which appear more common in less sophisticated, rural areas; increased risk is also observed in women living in households using municipal sources, which might be related also to environmental pollution (Greenlee *et al.,* 2003). On the other hand, the same study observed that actually residing on a farm, ranch or in a rural area had a significant protective effect, indicating that specific factors may affect female fertility, whereas rural life itself does not.

Certain situations of pesticide handling that lead to higher levels of direct exposures may be included among specific risk-factors for reproductive health that are present in a rural environment. In fact, de Cock *et al.* (1994) found that reduced fecundability ratio and longer time to pregnancy were associated with factors causing higher exposure to pesticides, namely: application of pesticides solely by the owner and low spraying velocity that, in turn, was associated with older spraying techniques. The study by Greenlee *et al.* (2003) identified an association of adverse reproductive outcomes in women with mixing and applying herbicides as well as with the use of fungicides. Greenhouse work is a peculiar type of agricultural work, entraining a continuous use of pesticides. In a large Danish study on time to pregnancy among female workers in flower greenhouses, the overall fecundability

rate did not differ between workers and referents. However, certain factors, possibly associated with increased exposure to chemicals, were consistently associated with a significant 20-30% reduction of fecundability, i.e., handling cultures many hours per week, spraying of pesticides, and not using gloves (Abell *et al.,* 2000).

Thus, attention should be paid to studies aiming at correlating adverse reproductive outcomes with specific groups of pesticides. An example is provided by the Ontario Farm Family Health Study, which was designed to assess retrospectively the potential adverse effects of exposure to pesticides on pregnancy. Exposure to phenoxy herbicides during the first trimester was generally not associated with increased risk of spontaneous abortion; on the other hand, a significant association was found for preconception exposure (Arbuckle *et al.,* 1999). Further investigation confirmed moderate but statistically significant increases in risk of early abortions for preconception exposures to phenoxy acetic acid herbicides, triazines, and any herbicide [odds ratios (OR) = 1.4-1.5 95% with 95% confidence intervals (CI) between 1.0-1.1 and 1.9-2.1]. For late abortions, a significantly elevated risk was observed for preconception exposure to glyphosate or thiocarbamates (ORs = 1.7-18 with; 95% CIs between 1.0-1.1 and 2.9-3.0). As for postconceptional exposure, specific pesticides increased the risk (all with a borderline statistical significance) only for the late abortions, namely, 2,4-D, dicamba, and again glyphosate and the phenoxy acetic acid herbicides. Older maternal age (> 34 years of age) was the strongest risk factor for spontaneous abortions; indeed, a most interesting finding of the study was the interaction between maternal age and chemicals. In the older age group there was a higher susceptibility to specific pesticides or pesticide groups, including thiocarbamates and overall fungicides which did not enhance abortion risk in younger women. The effect in the > 34 years age group was especially pronounced if combined exposure to two groups of chemicals occurred. In particular, a three-way interaction effect with maternal age was observed for preconceptional exposure triazines and thiocarbamates, as well as for carbaryl and 2,4-D, leading to a marked increase of risk (Arbuckle *et al.,* 2001).

Female reproductive dysfunctions (e.g., reduced fecundability and increased risk of miscarriage) may be associated also to exposure to pesticides of the male partner. In the Ontario Farm Family Health Study miscarriage risk increased with reported use of thiocarbamates, carbaryl as well as the combined of other compounds (atrazine, glyphosate, organophosphorus insecticides); mixing or applying yard herbicides was associated with preterm delivery (OR = 2.1, 95% CI 1.0-4.4) (Savitz *et al.,* 1997).

Arbuckle *et al.* (1999) reported an increased risk of abortion also for paternal exposure to phenoxy herbicides, especially if the male partner did not normally wear

protective equipment during application. The role of personal protective equipment was confirmed by a Finnish study on male greenhouse workers and time to pregnancy, where fecundability was decreased only in exposed workers that were inefficiently protected. In this study, increased time to pregnancy was specifically associated to exposure to pyrethroids as well as, to a lesser extent, to organophosphorus and carbamate insecticides (Sallmen *et al.,* 2003). Italian retrospective studies showed significantly increased risks of conception delay and spontaneous abortion among the spouses of high-exposure workers (greenhouse, applicators); in particular, exposure to chlorinated insecticides, triazines and benzimidazoles was implied (Petrelli and Figà-Talamanca, 2001; Petrelli *et al.,* 2000; Petrelli *et al.,* 2003). The increased risk was further supported following a careful evaluation of possible confounding factors such as age, education, smoking habits, etc. (Petrelli *et al.,* 2003). This could simply reflect an indirect exposure, e.g., through contaminated clothes or items or through unofficial help to the partner's work. On the other hand, this finding might reflect altered quality and integrity of semen; this might be expected, e.g., from the benzimidazole fungicides which are recognized testicular toxicants and can induce cytogenetic abnormalities of sperm (Amer *et al.,* 2003). Moreover, an inverse association between semen quality and levels of urinary biomarkers of exposure to different herbicides (e.g., alachlor, atrazine) and insecticides (e.g., diazinon) was observed recently in farmers from Missouri (Swan *et al.,* 2003). Therefore, evidence does exist for the possible association of reduced fecundability and pregnancy loss with certain situations of exposure to pesticides of the male partner.

Therefore, occupational epidemiology studies point to some group of pesticides specifically associated with an increased risk of reproductive dysfunction in women, namely, phenoxy acetic acid (Arbuckle *et al.,* 1999; Arbuckle *et al.,* 2001) and triazine herbicides (Savitz *et al.,* 1997), thiocarbamates and glyphosate (Arbuckle *et al.,* 2001). Concerning male-mediated effects, studies have indicated the exposure to pyrethroid, organophosphorus and carbamnate insecticides (Savitz *et al.,* 1997; Sallmen *et al.,* 2003) as well as, again, to phenoxy acetic acid (Arbuckle *et al.,* 1999) and triazine herbicides (Savitz *et al.,* 1997; Petrelli *et al.,* 2003). Triazines, thiocarbamates and same cholinesterase inhibitors are, in fact, potential EDC that may impair female reproductive function through different mechanisms (Cummings *et al.,* 2000; Narotsky *et al.,* 2001; Stoker *et al.,* 2003; Grunfeld and Bonefeld-Jorgensen, 2004; Hofmeister and Bonefeld-Jorgensen, 2004; Kojima *et al.,* 2004); on the other hand there is no evidence for endocrine activity of other pesticides groups that have been consinstently associated with reproductive impairment in women, such as glyphosate (Williams *et al.,* 2000). However, mechanisms other than endocrine disruption cannot be excluded, such as cytotoxicity or immune

deregulation; for instance, phenoxy acetic herbicides are associated with immunological changes in exposed workers (Faustini *et al.,* 1996). Nevertheless, the limited amounts of studies do not allow the identification of all pesticides that actually affect the reproductive health of female workers; most important, it is not yet feasible to have a quantitative risk assessment in different occupational situations.

3.2. Is there an effect resulting from exposure of the general population?

Residues in foods are likely to be a major source of exposure to pesticide residues of the general population. In Europe, the Pesticide Residue Monitoring Programme collects data from national monitoring programmes as well as remarks from the responsible bodies of the different EU countries on monitoring problems such as sampling strategies and validation of methods. Among compounds targeted by the programmes are several pesticides recognized as potentially hazardous for female reproduction such as dicofol, endosulfan, imazalil, etc. (European Commission, 2003a). According to the evaluation of the last report available, that of 2001, 96% of samples show either no detectable residues or residues below the maximum residue limits. On the other hand, 18% of samples show residues of more than one pesticide, with a significant trend to increase as compared to previous years. Overall, fungicides and insecticides continued to be the most commonly detected types of residues. Lettuce and strawberries were among common food items with a relatively higher frequency of residues above allowable limits, with special regard to the ethylene bisdithiocarbammate maneb and the benzimidazole benomyl, respectively (European Commission, 2003a; European Commission, 2003b). Estimates of consumer intake were always below the admissible daily intakes (ADI) for long-term exposures; however, the acute reference dose (ARfD, parameter for high short-term intakes, usually one-day or one-meal) was exceeded for the chlorinated insecticide endosulfan in lettuce (estimates for both adults and toddlers) and for the organophosphorus triazophos in apple (estimate for toddler), giving rise to some concern (European Commission, 2003a).

Pesticide monitoring programmes in vegetable foods, however important, may provide only rough estimates of actual whole dietary intakes; these should include also residues in foods of animal origin. Under this respect, an important issue is the transfer of pesticide residues in feeds to the food commodities of animal origin. The European Food Safety Authority (EFSA) is currently carrying out risk assessment of several feed contaminants. Among pesticides, a recent example is represented by camphechlor (also called toxaphene), a chlorinated insecticide including many

different congeners; camphechlor shows some bioaccumulation and may affect the endocrine and immune systems. The EFSA concluded that a proper risk assessment is still unfeasible, due to insufficient data on the exposure as well as on the toxicity and metabolism of the different congeners (European Food Safety Authority, 2005).

Risk assessment of dietary exposures needs to take into account groups with specific consumption patterns such as vegetarians and children (see, e.g., Fenske *et al.,* 2002a). Also, more attention should be paid to the additive intake of residues of compounds with similar mechanisms of toxicity and targets. A Danish study has evaluated the overall intake of cholinesterase-inhibiting insecticides (35 organophosphates and carbamates, altogether); estimates were based on the nation-wide food consumption survey performed in 1995 and pesticide residue-monitoring programmes from 1996-2001. The assessment was confined to vegetable foods, since no residues of organophosphorus compounds or carbamates were found in foods of animal origin during the monitoring programmes. Processing factors, such as reduction of pesticide levels by rinsing and peeling, were applied in the exposure assessment and the relative potency of chemicals was normalized through a “toxicity equivalence factor" approach. Neither ARfD nor ADI was exceeded in any of the compounds studied; in fact, the most conservative estimate indicated that the whole consumption reached up to 11% and 27% of the ADI for adults and children, respectively (Jensen *et al.,* 2003).

However important, diet is not the only way of exposure to pesticides for the general population. Living near sites where pesticides are used, manufactured or disposed may significantly increase the environmental exposure through air, water and soil. A recent study performed in the U.S.A. found a significant presence of chlorpyrifos, diazinon (organophosphates) and propoxur (carbamate) in maternal and cord blood of a mother-child cohort from a low-income New York area; the exposure was consistently associated with pest control (Whyatt *et al.,* 2005).

Since cholinesterase inhibitors are non-persistent compounds, these findings suggest that widespread and continuous exposure to pesticides of the general population might occur. Unfortunately, no data on biomonitoring of other currently used pesticides, such as the different groups with potential EDC activities, are yet available. Moreover, women in fertile age may represent a portion of the population that is particularly vulnerable to certain EDC and other pesticides; however, very few targeted studies are available. Villanueva *et al.* (2005) found only limited evidence for an association between atrazine levels in drinking water and small-for-gestational-age status, but not with preterm delivery or low birth weight. Cohn *et al.* (2003) investigated transplacental effects on the reproductive function of the

offspring; time to pregnancy was evaluated in a cohort of daughters in association with the serum levels of DDT and its metabolite p,p'DDE in preserved maternal serum samples drawn 1-3 days after delivery between 1960 and 1963. A negative association between fecundability and DDT levels was observed as well as a positive association with p,p'DDE levels; as regards this latter finding, the authors hypothesized an unexpected effect of the recognized antiandrogenic activity of the DDT metabolite.

Overall exposure to pesticides of general population is influenced by a number of factors related to diet and life styles as well as to the usage patters and environment persistence of different compounds. The great question yet to be answered is whether exposure of the general population may give rise to detectable health effects, considered also the presence of highly vulnerable individuals. Therefore, a most interesting development would be to correlate biomonitoring with early markers of toxicity.

4. Efforts at European level

The European Union is sensitized to the problem of pesticides. The European Community has developed a very comprehensive regulatory framework, Directive 91/414/EEC defining strict rules for the authorisation of pesticides, defined as "plant protection products" (PPPs). The Directive requires very extensive risk assessments for effects on health and environment to be carried out, before a PPP can be placed on the market and used, including the definition of maximum residue limits on food- and feedstuffs. As concerns the critical issue of risk analysis and food safety, the Panel on plant health, PPP and their residues is constituted within the EFSA, (http://www.efsa.eu.int), as a follow-up to the previous Scientific Committee on Plants. The Panel has to deal with questions on the safety of plant protection products for the user/worker, the consumer of treated products and the environment as well as for plant health. As all EFSA activities, this is focussed on risk assessment, whereas national governments and the European Commission are responsible for risk management and policy measures.

Moreover, in 2002, the European Commission adopted a Communication 'Towards a Thematic Strategy on the Sustainable Use of Pesticides' (European Commission, 2002). The Communication identifies several critical areas, objectives and possible solutions, including:

- encouraging good farming practice, integrated pest management, low-input or pesticide-free crop farming, in order to reduce dependence on chemical control

- support to epidemiological research on users and consumers

- technical improvements of application and protection equipment

- risks/benefits analysis for PPPs and alternatives

- improved controls on the use and distribution of pesticides

- review of old active ingredients

- implementing indicators to evaluate risk reduction programmes

- support to candidate countries, that now (2004) are becoming members of the European Union.

These measures, if effectively implemented, are likely to contribute to an improved level of environmental and health safety in the European Union. However, some problems deserve to be mentioned:

1. concerning food safety, the global food market with food items coming from countries using also old, toxic compounds no more allowed in Europe

2. concerning pesticide use for e.g., gardening or pest control, the possibility of a uncontrolled exposure in the household to which women in fertile age and children may be more vulnerable

3. concerning community health, the broad, still incompletely understood, range of factors potentially enhancing susceptibility to xenobiotics which include genetic background, metabolic imbalances, diet, other exposures, socio-cultural status, etc. There are no adequate data to characterize possible interactions (either detrimental or beneficial) or, most important, to evaluate their possible impact on policies for risk prevention and management.

Another major European initiative is the European Environment Health Strategy, aiming at revising the disease burden due to environmental factors in the European population. The Strategy does not deal specifically with pesticides; however, EDC are identified as priority. The integration among different information sources is

recommended, such as environmental, food residue and epidemiological monitoring programmes. As concerns female reproductive health, such disturbances as precocious puberty and endometriosis recognize an endocrine basis; therefore, it is underlined that the possible association with EDC deserves further attention (European Commission, 2004b).

5. Conclusions

The overall evidence points to female reproductive function as a sensitive target for certain groups of pesticides, in particular EDC. Several groups of compounds (chlorinated insecticides, thiocarbammates, triazines, triazoles, etc.) and mechanisms are already identified by experimental studies, although some problems still exist, namely:

- the elaboration of a comprehensive , integrated battery of *in vitro* screening tests in order to address and modulate further testing;

- a full appraisal of and *in vivo* parameters and protocols apt to characterize effects on female reproductive function, especially as regards long-term functional impairment following early exposures;

- the extrapolation of findings in animal models to humans, as endocrine control of certain phases of the reproductive cycle (e.g., parturition) show significant interspecies differences (European Commission, 1999).

Another interesting finding of experimental studies is that mechanisms altering the balance among different hormones may be as important as direct hormone agonism/antagonism, as suggested, e.g., for atrazine (Cooper *et al.,* 2000) or endosulfan (Hiremath and Kaliwal, 2002a; Hiremath and Kaliwal 2002b).

Data on occupational exposure to pesticides point to an association of pesticide exposure with reduced fecundability and increased risk of miscarriage. Studies consistently show that, rather than a generic exposure to pesticides or to agricultural work, risk is enhanced by specific groups of chemicals, including pesticides identified as EDC in experimental studies such as thiocarbammates, triazines and organophosphates (Arbuckle *et al.,* 2001). Moreover, risk is enhanced by behavioural factors, the most important being lack of efficient protective equipment. The contribution of other factors, such as maternal age, residential choices, personal habits, etc., must be regarded as important (Greenlee *et al.,* 2003); however, these may not be merely regarded as confounders, but as factors which can actually

interact with chemical exposure such as maternal age (Arbuckle *et al.,* 2001). Overall, the available information supports the elaboration of targeted preventive strategies, which might include closer monitoring, and possibly biomonitoring, of workers exposed to the most hazardous agents as well as proper campaigns for risk communication, education and training. Further information on the possible interaction between specific pesticides and other factors may be of value for prevention campaigns, as it can help to pinpoint groups or behaviours at higher risk which, otherwise, might be overlooked. Moreover, several epidemiological studies point out the prevention of exposure of the male partner to certain pesticides as a protective factor for the woman's reproductive health (Petrelli and Figà-Talamanca, 2001; Petrelli *et al.,* 2000; Petrelli *et al.,* 2003). From the public health point of view, the most relevant issue remains whether pesticide exposure may entail a risk for the general population.

According to the reports on pesticide residue monitoring programmes issued by the European Commission (European Commission, 2003a; European Commission, 2003b), exposure through vegetable foods is under control, although some areas of concern remain, such as the increased presence of samples with more than one type of residues. Also, improvements are desirable in order to harmonize and optimize sampling, analysis and reporting at European level (European Commission, 2003a). Greater attention, on the other hand, has to be paid to the monitoring of the contamination of foods of animal origin through feeds and pastures (European Food Safety Authority, 2005). It may also be desirable to integrate the information from food residue monitoring programmes and from relevant programmes for environmental monitoring, in order to implement more targeted and cost-effective strategies for prevention of risks to environment and public health. However, pest control may be another significant pathway of exposure of the general population, at least for certain pesticides, e.g., organophosphates (Whyatt *et al.,* 2005). Thus, more extensive data on biomarkers of exposure in association with predictors, or measures, of effect would help us understand whether and where there is a problem for the general population.

Finally, it will be of great interest to investigate the possible roles of pesticides in female reproductive dysfunctions other than miscarriage and delayed conception; an example could be endometriosis, which has been associated with increased exposure to persistent EDC (Rier, 2002). Investigations need, therefore, to develop novel approaches, integrating experimental and clinical research as well as developing and exploiting appropriate biomarkers.

Acknowledgements

This paper is elaborated within the frame of the following projects:

- I.S.S. grant C3CG "*Endocrine effects of substances potentially present as residues in foods of animal origin: innovative models for risk assessment*".

- I.S.S.-Foundation "S.Maugeri" grant H6E "*Endocrine disrupting or neurotoxic effects of industrial pollutants, herbicides and pesticides in the food chain*"

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ASSESSMENT OF FERTILITY AND OTHER HEALTH IMPACTS FOR SELECTED POLLUTANTS IN FLANDERS: *OPPORTUNITIES AND LIMITATIONS*

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Summary

In Belgium, the available information regarding the distribution and effects of endocrine disrupters is fragmentary, and has shortcomings that inhibit purposeful prevention and a policy of emission reduction.

In this paper, the fertility impacts of a selected group of substances are subjected to a health risk assessment. This allows the fragmentary nature of the available information to be determined systematically and functionally. The risk assessment focuses on human health risk related to indirect exposures, and is established for five groups of pollutants: dioxins (halogenated hydrocarbon), atrazine (pesticide), bisphenol A (phenols), ethynylestradiol (synthetic hormone) and the tributyltin compounds (biocide/insecticide).

The potential risks of the exposure to these endocrine disrupters are assessed using a 4-step procedure that entails: (1) identification of hazards, (2) effect assessment, (3) exposure assessment and (4) risk characterisation. To assess the exposure in the third step, data for a worst-case scenario (with maximum concentrations in the different environmental compartments), average values and data for a minimum scenario (with minimum concentrations) are used. For each substance, a human daily intake is calculated. This takes into account the exposure to air, drinking water and food. Results show that dioxins offer the greatest cause for health concerns in Belgium. A minimal daily intake of 2.56 pg teq/kg bw was calculated,

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 249–281.

corresponding to a higher risk of endometriosis and a decrease in lymphocytes. Bisphenol A and ethynylestradiol occur in concentrations that might constitute a health hazard. However, more research on the effects of both substances is required before final conclusions can be drawn. Overall, this study shows that the current environmental exposure to dioxins and probably bisphenol A and ethynylestradiol entails health risks for the population.

1. Introduction

Numerous industrial and agricultural chemicals have been disseminated worldwide during the past decades. Some of them have adverse developmental and reproductive effects on animals and humans. Concern about their presumed human adverse effects has grown both in the public at large and among policy makers.

In Belgium, the non-ferrous, ferrous and steel industries are major sources of dioxins, along with household heating and traffic. The contamination of humans by dioxins occurs mainly through such foods as meat, dairy products and fish (Liem *et al.,* 2000). Dioxins are associated with endometriosis, a higher cancer incidence and a decrease of thyroid functions (Eskenazi *et al.,* 2002).

Atrazine is a selective chlorotriazine herbicide used to inhibit the photosynthesis of annual weeds. In Belgium, atrazine is applied especially in maize and asparagus cultures (De Smet and Steurbaut, 2001). Reproductive effects and (stomach) cancer are potential adverse effects of exposure to environmental concentrations of atrazine (Van Leeuwen *et al.,* 1999).

Bisphenol A (BPA), a commonly used name for the synthetic chemical 2,2-(4,4-dihydroxydiphenyl)propane, is primarily applied in the manufacturing of polycarbonate and epoxy resins and as a flame retardant (Staples *et al.,* 2000). Exposure to BPA might result in reproductive effects and cancer (Vom Saal *et al.,* 1998).

The synthetic hormone 17α-ethynylestradiol (EE_2), which is used e.g. as an active component of the contraceptive pill (Maier *et al.,* 2001), is reactivated in Belgian water treatment installations. It is currently unclear how much of this active ethynylestradiol is consumed through drinking water. A higher risk of breast cancer and a protective effect on ovarian cancer after exposure to EE_2 have been reported (Althuis *et al.,* 2003).

Tributyltin compounds are mainly used as additives in paints to prevent the growth of marine organisms on fishing nets and ships. They are known to cause fetotoxic characteristics and skeleton damage during intra-uterine development.

This paper investigates the extent to which available data in Flanders (Northern Belgium) allow a risk assessment to be performed for the five priority pollutants: dioxins, atrazine, bisphenol A (BPA), 17α-ethynyl estradiol (EE_2) and tributyltin compounds (TBT). Additionally, it enables a preliminary quantification of the exposure of the Belgian population to the same substances. Results do not only point to health risks, and risks for reproductive health in particular; they also highlight important gaps in the basic knowledge, allowing environmental health risks to be assessed.

2. Risk assessment

The risk assessments of dioxins, atrazine, BPA, EE_2 and tributyltin compounds are based on the Technical Guidance Document (TGD) that supports the European Commission Directive 93/67/EEC on risk assessment for new notified substances and the Commission Regulation (EC) No 1488/94 on risk assessment for existing substances (Part I). This method allows the risk associated with a substance, e.g. a pollutant for the human population, to be calculated. The indirect exposure through air, water, soil and food of the Belgian population has been taken into account. Starting from the known health hazards, exposure and dose effect relationship, the risk can be quantified.

The risk assessment process includes four steps (Hens, 2001):

- hazard identification (step 1)
- effect assessment (step 2)
- exposure assessment (step 3)
- risk characterisation (step 4)

The risk assessments are limited to the indirect environmental exposure through food, drinking water and inhalation of air.

2.1. Hazard identification

The toxicological data provided by test systems, epidemiological studies, case reports and field observations are evaluated during the hazard identification.

In this study, the hazard identification was limited to four main adverse effects for humans: carcinogenicity, reproductive toxicity, neurotoxicity and immunotoxicity.

2.2. Effect assessment

This step identifies the concentrations at which the carcinogenic, reproductive, neurological or immunological effects occur in animals and humans. This part overviews NOAEL (No Observed Adversed Effect Level), where no adverse effects have been observed, and LOAEL (Lowest Observed Adversed Effect Level), the lowest concentrations linked to negative effects. In the risk characterisation step, the LOAEL-values are used to assess the potential risk for humans.

2.3. Exposure analysis

The exposure assessment involves (1) the specification of the exposed population, (2) the identification of the ways through which exposure occurs, and (3) the estimation of the exposure dose. Exposure analysis is achieved by identifying the concentrations of pollutants in the environmental compartments (air, water, soil) and other forms of exposure (food). Three exposure situations for each endocrine disrupting chemical are considered in this study: a worst-case scenario, an average scenario and a minimum scenario. Accordingly, the data for the highest, mean and lowest concentrations reported in the literature are used. The Belgian data were collected for concentrations of the pollutants in water, air and soil. Where no data for Belgium are available, data from neighbouring countries are used. All the available data are introduced into the model (TGD) (EC, 2003) to obtain an estimated human daily intake (EHDI) for each exposure pathway. Intake amounts are determined using standard defaults for indirect exposure of humans. The values are provided by the TGD. The total daily intake of the population is estimated, summarising the daily intake values for water, air, vegetables, cattle (meat and milk) and fish. This $EHDI_{tot}$ is calculated for an average person with a body weight of 70 kilograms.

2.4. Risk characterisation

This part of the analysis integrates the information collected from the first three steps to highlight the human effects linked to concentrations of pollutants present in the environment.

The estimated total daily intake ($EHDI_{tot}$) is compared with a reference dose (RfD). This value refers to the LOAEL and includes a safety margin. At this exposure level, no risk of adverse effects has to be expected. In practice, the RfD is obtained by dividing the LOAEL levels (determined during the effect assessment) by an uncertainty factor of 90, which compensates for the extrapolation of data for laboratory mammals to mammals. A factor of 100 is used to extrapolate data of non-mammalian species.

An $EHDI_{tot}$ higher than the RfD indicates an elevated risk of the described adverse effects for the Belgian population.

3. Risk assessment of dioxins

3.1. Hazard identification

Endocrine disrupters imitate or block natural hormones, especially at crucial moments of the *in utero* life and postnatal development. 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) activates the aryl hydrocarbon receptor (AhR) by forming a ligand. The receptor will separate from its molecular chaperones and penetrate into the nucleus. This causes a dimerisation with the Arnt (Ah receptor nuclear translocator) that modulates the capacity to interact with DNA and induces the transcription of target genes. The P450 iso-enzymes CYP1A1 and CYP1A2 are activated and influence the steroid housekeeping of estrogens, testosterone and the thyroid stimulating hormone (TSH) (ATSDR, 2003).

The carcinogenic effects are based on non-genotoxic mechanisms, where 2,3,7,8-TCDD acts as a cancer-promoter. In most of organs, cancer will be initiated by an AhR-mediated transcription, which leads to oxidative DNA-lesions.

Dioxins also decrease the immune response. 2,3,7,8-TCDD changes the amount of T-cell populations and decreases the number of activated antibodies.

3.2. Effect assessment

To assess the effects caused by dioxins, dose-response relations for different effects are reviewed in test animals and - whenever available - in humans.

3.2.1. Dose-response relations in animals

Table 18 provides an overview of the LOAELs and NOAELs for dioxins in animal experiments. The table lists the available data for cancer, neurotoxic and immunological endpoints.

Table 18. Dioxins: Dose-response relations in animal studies

Effect	Animal species	NOAEL*	LOAEL*	Organ/type of effect	References
Cancer	Rat	1	100	Lung, nose, tongue carcinoma	Kociba *et al.,* 1978
Reproduction	Rat (m)	-	0.126	Decrease number spermatozoa	Gaudet, 2001
	Rhesus monkey (f)	-	0.126	Endometriosis	Rier *et al.,* 1993
	Rhesus monkey	-	0.642	Fetotoxicity	INSERM, 2000
Neurological	Rhesus monkey	-	0.15	Cognitive influence on offspring: decrease object recognition, decrease learning behaviour	Schantz *et al.,* 1989
Immunological	C57BL/5	-	0.126	Increase sensitivity to viral infections	IARC, 1997
	Marmot monkey	-	0.135	Decrease lymphocytes	IARC, 1997

* = *NOAEL and LOAEL expressed in ng/kg bw/day, unless otherwise mentioned* (m) = male, (f) = female

3.2.2. Dose-response relations in humans

Different studies established dose-effect relations in humans after exposure to dioxins. A lifetime intake of 0.006 pg TEQ dioxins/kg bw/day causes an increased unit cancer risk of 1 in 1,000,000 persons (SCF, 2000; Van Larebeke *et al.,* 2001).

Mocarelli *et al.* (1996) analysed women who were exposed to dioxins after the Seveso accident. They found a change in sex ratio in favour of girls. The interpretation is that male embryos are more susceptible to hormonal deregulation and that the peak of testosterone, needed for the development of a male, is destroyed by the exposure to dioxins. Concentrations of more than 118 pg TEQ TCDD/g serum lipids in the man during conception are related to significant change in the sex ratio, favouring the birth of females (Mocarelli, 2000; Eskenazi, 2002).

A body burden of 25 µg TEQ/kg bw in a woman is related to decreased estradiol and increased progesterone levels. These changes could cause secondary amenorrhea (SCF, 2000).

Immunological effects involve: (-) a decrease of the plasma values of IgG in women with a body burden of 608 ng TEQ/kg (SCF, 2000), (-) babies who are fed with breast milk containing dioxin concentrations of 30.75 – 76.43 pg TEQ/g fat show changes in the thyroid status: increased concentrations of thyroid stimulating hormone (TSH) and decreased concentrations of thyroxine (T_4) (Koopman-Esseboom *et al.,* 1994).

3.3. Exposure assessment

Where possible, the exposure data are collected for Flanders. If data are lacking for specific compartments, the assessment is based on data from the Netherlands and/or foreign countries.

A concentration of 5 fg TEQ/l in drinking water (Travis and Hattermeyer-Frey, 1987) was used for the three situations (worst-case, average and minimal exposure scenario). An estimated human daily intake (EHDI) of 0.14 fg TEQ/l drinking water could be calculated (see table 27). Air concentrations for Flanders (MIRA-T, 2001) are used to calculate the daily intake of dioxins through air, which varies between 6.30 and 249.10^{-3} pg TEQ/m^3 (Table 19).

Table 19. Dioxins: Concentrations in environmental compartments for the three scenarios

Scenario	C_{air} (10^{-3} pg TEQ/m^3)	C_{water} (10^{-3} pg TEQ/l)	C_{soil} (10^{3} pg TEQ/kg dw)
Minimum	6.30	8.03	3.90
Average	75.78	11.50	9.80
Worst-case	249.00	14.98	27.20

The concentrations of pollutants in surface water allow the intake by cattle and fish and their presence in vegetables to be assessed. Surface water concentrations are determined by the use of sediment concentrations for the Netherlands (in the Western Scheldt) (Liem *et al.,* 1993): $8.03.10^3$ pg TEQ/kg dw (minimum scenario), $11.50.10^3$ pg TEQ/kg dw (mean scenario) and $14.98.10^3$ pg TEQ/kg dw (worst-case scenario).

The water soil concentrations in Flanders range from $3.9.10^3$ pg TEQ/kg dw (minimum scenario) over $9.8.10^3$ pg TEQ/kg dw (average scenario) to $27.2.10^3$ pg TEQ/kg dw (worst-case scenario) (Koppen *et al.,* 2002) (Table 20). These data are used to estimate the concentrations in plants. The values of dioxins in plants provide the estimation for the concentrations in vegetables.

Concentrations in cattle are calculated by combining concentrations in surface water, air and soil. To estimate concentrations of dioxins in meat and milk, the intake of dioxins in cattle is calculated and is transferred to meat and milk using bio transfer factors.

Analysis of human breast milk was carried out in Brussels and Liège between 1988 and 1993. The mean concentration found was 34.4 pg TEQ/g milk fat.

The total estimated human daily intakes ($EHDI_{tot}$) are shown separately in Table 21 for the different environmental exposure situations (worst-case, average and minima). A total EHDI for a person of 70 kilos, is situated between 2.56 and 14.81 pg TEQ/kg bw/day, with an average of 5.79 pg TEQ/kg bw/day. The most important source of dioxin exposure for the Belgian population is the meat and milk consumption, and to some extent the fish consumption.

Table 20. Dioxins: Estimated human daily intakes for three scenarios in: 10^{-3} pgTEQ/kg bw/day

Scenario	EHDI drink water	EHDI inhalation	EHDI fish	EHDI vegetables	EHDI meat	EHDI milk	EHDI total (*)
Minimum	0.14	1.35	608.58	1.21	1223.27	720.95	2555.50
Average	0.14	16.24	871.57	3.05	3081.14	1819.19	5791.33
Worst-case	0.14	53.36	1135.31	8.47	8564.71	5047.67	14809.66

(*) Total estimated human daily intake and summarises the EHDIs from each compartment.

3.4. Risk characterisation

3.4.1. Extrapolation of animal LOAEL values to reference doses (RfD)

The reference doses (RfD) are based on LOAELs to deduce effects related to the exposure of the humans to dioxins.

Based on the EC (2003), the LOAEL in animals is divided by an uncertainty factor of 90 to obtain the RfD. Next, the estimated human daily intake (EHDI) is compared to the reference dose RfD for each effect. When the EHDI exceeds the RfD, this means that the environmental dioxin concentrations might have health consequences in Flanders.

Comparing EHDI values with RfDs for the three scenarios points to a risk of a decrease in the total number of sperm cells and the development of endometriosis (Table 21).

In addition, immune suppression can occur. A decrease in lymphocytes is associated with an increased sensitivity to viral infections. The highest maternal daily concentration (14.81 pg TEQ/kg bw/day) is associated with delayed development of the baby (after exposure of the embryo) and a higher risk of fetotoxicity and spontaneous abortion.

Table 21. Dioxins: Calculation of RfD and comparison with the $EHDI_{tpt}$, expressed as pg TEQ/kg bw/day). The shadowed part of the table highlights scenario values that exceed the corresponding RfD (EHDI > RfD).

Effect	LOAEL	RfD	Minimum scenario	Average scenario	Worst-case scenario
Decreased number of spermatozoa	126[a]	1.40	2.56	5.79	14.81
Endometriosis	126[b]	1.44	2.56	5.79	14.81
Fetotoxicity	642[c]	7.13	2.56	5.79	14.81
Decrease object recognition	150[d]	1.67	2.56	5.79	14.81
Increased sensitivity for viral infections	126[e]	1.40	2.56	5.79	14.81
Decreased number of lymphocytes	135[e]	1.50	2.56	5.79	14.81

a = Gaudet (2001), b = Rier et al. (1993), c = INSERM (2000), d = Schantz et al. (1989), e = IARC (1997)

3.4.2. Risk characterisation based on epidemiological studies

The number of cancers on a lifetime basis in Flanders will increase. A lifetime exposure to 2.56 pg TEQ/kg bw/day will result in an estimated number of 2539 extra cancer cases for a population of 5 950 000. For a lifetime exposure to 5.79 pg TEQ/kg bw/day and to 14.81 pg TEQ/kg bw/day, respectively 5 742 and 14 687 new cases are calculated for 5 950 000 habitants.

Exposure of the father to 14.81 pg TEQ/kg bw/day during the conception period is related to a shift in the sex ratio towards female descendants.

Exposure to 34.4 pg TEQ/kg fat in breast milk will induce higher TSH-concentrations and a lower T_4 values in the serum of babies. These observations can be linked to an increased risk of thyroid cancer (Kohn *et al.,* 1996).

4. Risk assessment for atrazine

4.1. Hazard identification

Atrazine inhibits the production of cytokines (interferon INF-γ, interleukin IL-5 and TNF-α) and the cell proliferation (Hooghe *et al.,* 2000). It stimulates the estrogenic activity by induction of aromatase, an enzyme that converts androgen into oestrogen. Due to the induction of aromatase, atrazine influences the reproductive system by respectively decreasing and increasing the secretions of the neurotransmitters norepinefrine and dopamine. It results in the suppression of the luteinizing hormone (LH) and prolactin (PRL) (Cooper *et al.,* 1998 and 2000; Crain *et al.,* 1997; Sanderson *et al.,* 2000).

4.2. Effect analysis

4.2.1. Dose-response relations in animals

Table 22 provides an overview of the most important effects of atrazine in animals and the related LOAEL values. Data related to NOAEL values are not available.

4.2.2. Dose-response relations in humans

Humans show an increased risk of developing stomach cancer when exposed to concentrations of 50 to 649 ng/l of atrazine in drinking water on a lifetime basis (Van Leeuwen *et al.,* 1999).

Table 22. Dose-response relations for atrazine in animal studies

Effect	Animal species	LOAEL*	Organ/type of effect
Carcinogenicity	SD rat[a]	20	Breast tumour
Reproductive effects	South-African claw frog (f)[b]	21 µg/l in water	Resorption of primary and secondary ovaries by exposure during sexual differentiation
	South-African claw frog (m)[b]	21 µg/l in water	Decrease testicular volume, testicular resorption & aplasy
	SD & LE rat[c]	75	Decrease of LH and PRL concentrations
	Pig[d]	1	Alteration in oestrogen cycle Decrease E_2-concentration: delay of oestrogen cycle
	Pig [e]	2	Ovarian follicular cysts Persistency of corpus luteum

a = Stevens et al. (1994), b = Travera-Mendoza et al. (2002), c = Cooper et al. (1996), d = Gojmerac et al. (1999), e = Gojmerac et al. (1995)

** = NOAEL and LOAEL expressed as mg/kg bw/day, unless otherwise mentioned*

(m) = male, (f) = female

4.3. Exposure assessment

A concentration of 5 µg/l in drinking water (worst-case scenario and average scenario) and a value of 0,1 µg/l (minimum scenario) are used (Dejonckheere *et al.,* 1996 a, b) to calculate an EHDI of 0.14 µg/l and 0.027 µg/l drinking water (Table 23). Rainwater concentrations in Belgium are collected to estimate air concentrations (Quaghebuer and De Wulf, 1999).

Table 23. Atrazine: Concentrations in environmental compartments for three scenarios

Scenario	C_{drink} (µg/l)	C_{air} (ng/m^3)	$C_{surface\ water}$ (µg/l)	$C_{soil\ water}$ (ng/l)
Minimum	0.10	0.04	10.00	100.00
Average	5.00	0.04	10.00	205.00
Worst-case	5.00	0.11	69.00	310.00

Atrazine in surface water was determined in 2000 and 2001 by the Flemish Environmental Agency (VMM, 2002): 10 μg/l for the average scenario and 69 μg/l for the worst-case scenario were found. However, no data for atrazine in soil water are available. Therefore, data for the UK are used (Clark *et al.,* 1981).

The total estimated human daily intakes ($EHDI_{tot}$) are shown in Table 24. Data for the three different environmental exposure situations (worst-case, average and minima) are provided. The sum of all individual EHDIs results in a total EHDI. For a person of 70 kilos, this value ranges between 0.014 and 4.449 μg/kg bw/day, with an average of 0.77 μg/kg bw/day. The Belgian population is primarily exposed to atrazine by fish consumption (Table 24).

Table 24. Atrazine: Estimated human daily intakes for three scenarios

	Unit	Minimum scenario	Average scenario	Worst-case scenario
EHDI drinking water	mg/kg bw/day	0.027	0.143	0.143
EHDI inhalation	10^{-3} □g/kg bw/day	0.009	0.009	0.024
EHDI fish	□g/kg bw/day	0.006	0.622	4.290
EHDI vegetables	□g/kg bw/day	5.030	10.260	15.600
EHDI meat	10^{-3} □g/kg bw/day	1.070 x10^{-3}	0.029	0.200
EHDI milk	10^{-3} □g/kg bw/day	6.310 x10^{-4}	0.017	0.120
EHDI total (*)	□g/kg bw/day	0.014	0.770	4.449

(*) Total estimated human daily intake and summarises the EHDIs from each compartment.

4.4. Risk characterisation

4.4.1. Extrapolation of animal LOAEL values to reference doses (RfD)

Comparison of the EHDI values with the RfDs for observed health effect in animals shows that the amount of egg cells and the testicular weight is expected to decrease at daily intake values of 0.770 μg/kg bw/day (average scenario) and 4.449 μg/kg bw/day (worst-case scenario) (Table 25). These results point to a decrease in fertility for both genders.

4.4.2. Risk characterisation based on epidemiological studies

The standard for atrazine in drinking water in Belgium has been established at 100 ng/l. This means that the Belgian population already has a potential risk of developing stomach cancer as Van Leeuwen *et al.* (1999) demonstrated an increased number of cancers related to an oral exposure to 50 – 649 ng atrazine /l of drinking waters.

Table 25. Atrazine: Calculation of RfD and comparison with the $EHDI_{tpt,}$ expressed as mg/kg bw/day). The shadowed part of the table highlights scenario values that exceed the corresponding RfD (EHDI > RfD).

Effect	LOAEL	RfD	Minimum scenario	Average scenario	Worst-case scenario
Breast tumour	20 [a]	222.22	0.014	0.770	4.449
Decrease of number egg cells and testicular volume	0.021[b]	0.21	0.014	0.770	4.449
Decrease of LH-PRL concentration	75[c]	833.33	0.014	0.770	4.449
Decrease of E_2 concentration, alteration in oestrogen cycle	1[d]	11.11	0.014	0.770	4.449
Ovarian follicular cysts	2[e]	22.22	0.014	0.770	4.449

a = Stevens et al. (1994), b = Travera-Mendoza et al. (2002), c = Cooper et al. (1996), d = Gojmerac et al. (1999), e = Gojmerac et al. (1995)

5. Risk analysis for bisphenol A (2,2-(4,4-dihydroxydiphenyl) propane)

5.1. Hazard identification

Bisphenol A (BPA) mimics the action of the female hormone oestrogen and binds on the oestrogen receptors ERα and ERβ. BPA reacts with the cytochrome P450-depending mono-oxygenase by inhibition of the activation of 16β-hydroxylase and testosterone-2α-hydroxylase in the microsomes of the liver. BPA changes the expression of the receptors of oestrogen and androgen and features an anti-androgen activity by inhibition of the production of dihydrotestosterone. It also has a role in the induction of the prolactin gene (Danzo, 1998; Fernandez *et al.,* 2001; Markey *et al.,* 2001b; Nativelle-Serpentini *et al.,* 2003; Rubin *et al.,* 2001).

5.2. Effect analysis

5.2.1. Dose-response relations in animals

Table 26 summarizes the most important endocrine disrupting effects of bisphenol A in animals. For each effect, NOAEL and LOAEL values are indicated in as much as they have been published in the literature.

Table 26. Bisphenol A: Dose-response relations in animal studies

Effect	Animal	Duration	NOAEL*	LOAEL*	Organ/type of effect
Reproduction	Rats[a]		2	100	Decreased testis weight
	F344 rats[b]	3 days		300	Increased uterus weight
	Rats[b]	*In utero*		300	Increased prostate weight
	Male mouse[a]		2	20	Decreased daily sperm production
	Mouse [c]	During days 11 & 17 of the gestation		2.4	Early puberty, alterations in reproductive functions, increase prostate weight, decrease epididymal weight
Neurological	Male [d]	During pregnancy and breastfeed		40	Increased behaviour of protection
	F344 rats[e]			200	Increased serum prolactin concentrations

a = Milman et al. (2002), b = Fernandez et al. (2001), c = Vom Saal et al. (1998), d = Farabollini et al. (2002), e = Markey et al. (2001a)

** LOAEL and NOAEL values are expressed as μg/kg bw/day, unless otherwise mentioned*

5.2.2. Dose-response relations in humans

BPA had been detected in fluids of vegetables packed in cans, in concentrations ranging between 0 and 23 μg per can. The highest concentration allowed the proliferation of MCF-7 cells to be induced *in vitro* (Markey *et al.,* 2001a).

The concentrations of BPA range between 0.3 and 18.9 ng/ml in blood samples of pregnant women (at 32nd and the 41th week of pregnancy). These concentrations are similar to the concentrations that cause toxicological effects on fertility organs in the male and female offspring of rats and mice.

5.3. Exposure assessment

BPA is not measured in drinking water in Belgium. The data for the Netherlands (minimum scenario) and the Czech Republic (worst-case scenario) are used instead (Vethaak *et al.,* 2002; Poustka *et al.,* 2002) (Table 27).

Data for BPA concentrations in air are not available for the whole of Europe. Only for Japan have data for air concentrations been published: 0.003 μg/m^3 (minimum scenario), 0.036 μg/m^3 (average scenario) and 0.05 μg/m^3 (worst-case scenario) (Yamamoto *et al.,* 1999). BPA has been found in the surface water in Belgium and the Netherlands. The pattern of the BPA-distribution in the Meuse River is irregular. The highest concentration (0.58 μg/ l) was found in Liège (BKH, 2000; Belfroid *et al.,* 2002).

No data for soil water concentrations are available for Flanders. Data for Germany are used instead (Wenzel *et al.,* 1998): 81.08 μg/l (worst-case), 11.55 μg/l (average) and 0.016 μg/l (minimum scenario).

Table 27 reviews the environmental concentrations that were used in the three scenarios.

Table 27. Bisphenol A: Concentrations in environmental compartments for three scenarios.

Scenario	C_{drink} (ng/l)	C_{air} (ng/m^3)	$C_{surface\ water}$ (ng/l)	$C_{soil\ water}$ (ng/l)
Minimum	12.00	2.90	3.50	16.00
Average	106.00	36.00	35.00	11,548.00
Worst-case	200.00	50.00	580.00	81,080.00

The total estimated human daily intakes ($EHDI_{tot}$) are represented in Table 28. The values are calculated for the three different environmental exposure situations (worst-case, average and minimal). The sum of all individual EHDIs gives a total EHDI, for a person of 70 kilos, ranging between $3.18.10^{-3}$ and 7.51 μg/kg bw/day, with an average of 1.07 μg/kg bw/day.

The Belgian population is primarily exposed to bisphenol A through the consumption of vegetables.

Table 28. Bisphenol A: Estimated human daily intakes for three scenarios

	Unit	Minimum scenario	Average scenario	Worst-case scenario
EHDI drinking water	10^{-3} µg/kg bw/day	0.34	3.03	5.71
EHDI inhalation	10^{-3} µg/kg bw/day	0.62	7.71	10.71
EHDI fish	10^{-3} µg/kg bw/day	0.76	7.61	126.19
EHDI vegetables	10^{-3} µg/kg bw/day	1.45	1,048.41	7,360.85
EHDI meat	10^{-3} µg/kg bw/day	$1.13.10^{-4}$	0.73	5.12
EHDI milk	10^{-3} µg/kg bw/day	$6.67.10^{-3}$	0.43	3.02
EHDI total (*)	µg/kg bw/day	$3.18.10^{-3}$	1.07	7.51

(*) Total estimated human daily intake and summarises the EHDIs from each compartment.

5.4. Risk characterisation

5.4.1. Extrapolation of animal LOAEL values to reference doses (RfD)

Exposure to average concentrations of 1.07 µg/kg bw/day is reported to decrease the daily sperm production, and even lead to infertility. Earlier puberty, the alteration of the reproductive functions, as well as an increased behaviour of protection have also been reported. The daily intake of the highest concentration (7.51 µg/kg bw/day) is associated with a higher risk of change in the size of the testis, a weight increase of the prostate and uterus, and may have an influence on growth and reproduction. Regarding the neuroendocrine effects, the prolactin concentration in the serum can rise. Intake of the minimum concentration ($3.18.10^{-3}$ µg/kg bw/day) will not trigger adverse effects.

Table 29. Bisphenol A: Calculation of RfD and comparison with the $EHDI_{tot}$, expressed as µg/kg bw/day. The shadowed part of the table highlights scenario values that exceed the corresponding RfD (EHDI > RfD)

Effect	LOAEL	RfD	Minimum scenario	Average scenario	Worst-case scenario
Alteration in growth and reproduction	640[a]	6.4*	$3.18.10^{-3}$	1.07	7.51
Infertility	1[b]	0.01*	$3.18.10^{-3}$	1.07	7.51
Decreased testes size	100[c]	0.11	$3.18.10^{-3}$	1.07	7.51
Increase of prostate and uterus weight	300[b]	3.33	$3.18.10^{-3}$	1.07	7.51
Decreased daily sperm production	20[c]	0.22	$3.18.10^{-3}$	1.07	7.51
Earlier puberty/change in reproductive functions	2.4[d]	0.027	$3.18.10^{-3}$	1.07	7.51
Increased behaviour of protection	40[e]	0.44	$3.18.10^{-3}$	1.07	7.51
Increased PRL concentration	200[f]	2.22	$3.18.10^{-3}$	1.07	7.51

a = Milman et al. (2002), b = Fernandez et al. (2001), c = Vom Saal et al. (1998), d = Farabollini et al. (2002), e = Markey et al. (2001)

5.4.2. Risk characterisation based on epidemiological studies

Völkel *et al.* (2002) showed that an important part of the daily intake of BPA is excreted with the urine after glucuronisation in the liver to BPA-glucuronide. This mechanism ensures that BPA does not reach high concentrations in the human body and that BPA-glucuronide does not disrupt the hormonal activity. Glucuronisation can be seen as a deactivation reaction, resulting in very small quantities of BPA being available for binding on the receptors. The possible risks of adverse effects based on hormonal activity of BPA could therefore not be predicted by a simple extrapolation of the data provided by rodent tests. Differences in toxic kinetics between rodents and humans have to be taken into account. BPA has a slow excretion rate in rodents because of the enterohepatic circulation, which has not been found in humans.

According to Haighton *et al.* (2002), no human epidemiological study has been found to evaluate a carcinogenic potential of BPA. Low dose administration studies in rodents often show contradictory results. For a given concentration, one study

will detect no effect, while another will estimate that the same specific concentration has a significant effect.

6. Risk assessment of ethynylestradiol (17α-ethinyl-1,3,5(10) oestratriene-3,17-diol)

6.1. Hazard identification

Ethynylestradiol (EE_2) interacts with the estrogenic receptor ER and imitates the endocrine functions of estradiol and progesterone. These hormones are female fertility hormones that regulate a range of biosynthetic and metabolic events. Exposure to low concentrations in the environment might disrupt the normal endocrine and reproductive functions, especially when the exposure occurs during critical periods of the development. The capacity of EE_2 to induce higher *in vivo* estrogenic effects than estradiol is caused by the 17α-ethinyl group of EE_2. This group reduces the breakdown rate of the steroid and induces a longer half-life for EE_2. EE_2 can cause endocrine disruption even in concentrations that are 10 times lower than those of natural hormones (Routledge *et al.,* 1998a).

EE_2 found in waste water effluents represents the main cause of environmental exposure to oestrogens (Larsson *et al.,* 1999; Foran *et al.,* 2002).

6.2. Effect analysis

6.2.1. Dose-response relations in animals

Table 30 lists the health effects caused by EE_2 in animals and the corresponding NOAEL and LOAEL values. NOAEL and LOAEL values are expressed as μg/kg bw/day, unless otherwise mentioned (m) = male.

6.2.2. Dose-response relations in humans

Few data exist on the carcinogenicity of EE_2 in humans. Oral contraceptives containing EE_2 are associated with a higher incidence of (benign) liver tumours and breast cancer.

Women, who use contraceptives containing over 35 μg EE_2/each pill, have a higher risk of breast cancer than women exposed to lower concentrations of EE_2. On the other hand, Royar *et al.* (2001) found that low dose contraceptives with less than 35 μg EE_2 will protect the body against the development of ovary cancer.

Table 30. Ethynylestradiol: Dose-response relations in animal studies

Effect	Animal species	NOAEL*	LOAEL*	Organ/type of effect
Global	Rabbit[a]	-	1.5	Anorexia
Cancer	Rats[a]	-	300	Increase adenomas and cell plasma
Reproduction	Rats (m) [b]	10	50	Decrease weight of prostate, pituitary gland, increase testis weight
	Mouse *in utero* (m) [c]	-	0,002	Decrease daily sperm production during adolescent period
	Female rats[b]	50	200	Decrease ovary weight, increase uterine weight
	Zebra fish [d]	-	1,67 ng/l	Induction of vitellogenin and decrease in fertilization success
Neurological	Elrits[e]	-	2 ng/l	Decrease in aggressive behaviour

a= Maier et al. (2001), b = Yamasaki et al. (2002), c = Thayer et al. (2001), d = Segner et al. (2003), e = Majewski et al. (2002)

6.3. Exposure assessment

EE_2 is detected in the effluents of sewage plants in different countries. Women who use contraceptive pills containing EE_2 excrete this EDC in urine and faeces in the form of its hydroxy metabolite, which is not an oestrogen. However the hydroxy metabolite of EE_2 is activated again in the sludge to an estrogenic active product that binds to the ER, and becomes estrogenic (Vethaak *et al.,* 1996; Larsson *et al.,* 1999; Foran *et al.,* 2002).

This assessment is based on data for drinking water in the Netherlands and Germany (Christensen, 1998; Vandenbergh *et al.,* 2000; Vethaak *et al.,* 2002): 0.11 ng/l (minimum); 6.4 ng/l (average) and 34.20 ng/l (worst-case scenario) (Table 31). No information on air concentrations is available for Belgium. As EE_2 concentrations in air are negligible, this has no influence on the result of the risk assessment for ethynylestradiol.

Ethynylestradiol is only occasionally measured in surface waters in Belgium. Therefore, the concentration in German effluents (64 ng/l) is used for the worst-case

scenario. For the minimum scenario, the lowest value found in the Netherlands is used (0.02 ng/l). (Belfroid *et al.,* 1999; Lai *et al.,* 2002; Vandenbergh *et al.,* 2000; Vethaak *et al.,* 2002). Water soil concentrations (Table 31) are based on soil concentrations reported in the UK (Lai *et al.,* 2002): 0.50 ng/kg dw (minimum), 3.27 ng/kg dw (average) and 9.81 ng/kg dw (worst-case scenario).

Table 31. Ethynylestradiol: Concentrations in environmental compartments for three scenarios.

Scenario	C_{drink} (ng/l)	$C_{surface\ water}$ (ng/l)	C_{soil} (ng/kg dw)
Minimum	0.11	0.02	0.50
Average	6.40	1.00	3.27
Worst-case	34.20	64.00	9.81

The total estimated human daily intakes ($EHDI_{tot}$) for the 3 scenarios are listed in the Table 32 The sum of all individual EHDIs estimates a total EHDI for a person of 70 kilos. The value ranges between 0.013 and 28.63 ng/kg bw/day, with an average of 0.62 ng/kg bw/day. The highest contribution to the exposure to ethynylestradiol is the consumption of fish and to some extent also to drinking water. This is confirmed by Christensen *et al.* (1998).

Table 32. Ethynylestradiol: Estimated human daily intakes for the three scenarios. The shadowed part of the table is the total estimated human daily intake and summarises the EHDIs from each compartment.

Scenario	EHDI drinking water (ng/kg bw/day)	EHDI fish (ng/kg bw/day)	EHDI vegetables (ng/kg bw/day)	EHDI meat (ng/kg bw/day)	EHDI milk (ng/kg bw/day)	EHDI total (ng/kg bw/day)
Minimum	$3.140.10^{-3}$	$8.600.10^{-3}$	$1.50.10^{-3}$	$2.940.10^{-6}$	$1.730.10^{-6}$	0.013
Average	0.183	0.430	0.010	$4.340.10^{-5}$	$2.560.10^{-5}$	0.620
Worst-case	0.980	27.620	0.029	$1.830.10^{-3}$	$1.080.10^{-3}$	28.630

6.4. Risk characterisation

6.4.1. Extrapolation of LOAEL values to reference doses (RfD) (EC, 2003)

Comparison of the EHDIs to the RfDs for EE_2 allows increased risks in humans to be identified. A daily intake of 28.63 and 0.62 ng/kg bw/day is liable to decrease the

daily sperm production during adolescence and the fertilization success of women. In addition, aggressive behaviour might decrease. The highest concentration (28.63 ng/kg bw/day) may possibly cause anorexia. Other effects dealt with in the risk assessment are not likely to occur.

Table 33. Bisphenol A: Calculation of RfD and comparison with the $EHDI_{tot}$, expressed as µg/kg bw/day. The shadowed part of the table highlights scenario values that exceed the corresponding RfD (EHDI > RfD)

Effect	LOAEL	RfD	Minimum scenario	Average scenario	Worst-case scenario
Increase of adenomas and cell plasma	300[a]	3333.33	0.013	0.62	28.63
			230.02**	230.73**	262.40**
Anorexia	1.5[a]	16.67	0.013	0.62	28.63
			230.02**	230.73**	262.40**
Decrease of prostate, seminal vesicles and pituitary weights/ increase of testis weight	50[b]	555.56	0.013	0.62	28.63
			230.02**	230.73**	262.40**
Decrease of daily sperm production during adolescence	2.10^{-3}[c]	0.022	0.013	0.62	28.63
			230,02**	230,73**	262,40**
Decrease of ovary weight, increase of uterine weight	200[b]	2222.22	0.013	0.62	28.63
			230.02**	230.73**	262.40**
Decrease in fertilization success (w)	$1.67.10^{-3}$ [d]	0.017*	0.013	0.62	28.63
			230.02**	230.73**	262.40**
Decrease in aggressive behaviour	2.10^{-3} [e]	0.020*	0.013	0.62	28.63
			230.02**	230.73**	262.40**
Suppressed sexual behaviour (m)	6[f]	66.67*	0.013	0.62	28.63
			230.02**	230.73**	262.40**

a= Maier et al. (2001), b= Yamasaki et al. (2002), c= Thayer et al. (2001) , d= Segner et al. (2003), e= Majewski et al. (2002), f= Halldin et al. (1999)

(w) = women, (m) = men, RfD = LOAEL/90

** RfD = LOAEL/100*

***based on women of 60 kg and users of oral contraceptives with an average of 35 µg/pill.*

For EE_2, the total EHDIs are also calculated for a person of 60 kg, corresponding to the average weight of women. These results in the following values: 0.02 ng/kg bw/day (minimum), 0.73 ng/kg bw/day (average) and 32.40 ng/kg bw/day (worst-case scenario). The daily EE_2 intake of non-pregnant women who use oral contraceptives with a mean concentration of 35 µg/day is also considered to estimate

the potential risk incurred by these women. Carr and Griffin (1998) reported that 60% of the EE_2 is excreted with the urine after 24 hours. Consequently, oral contraceptive users are possibly exposed to 40% * 35 μg/day = 230 ng/day more than non-users. This concentration, added to the EHDI for women, gives total EHDIs of 230.02 ng/kg bw/day (minimum), 262.40 ng/kg bw/day (average) and 230.73 (worst-case) ng/kg bw/day, representing the concentrations presented by women who use oral contraceptives. This results in concentrations (Table 33) that are much higher than the initially calculated EHDI; so the risk of the described effects is therefore increased.

6.4.2. Risk characterisation based on epidemiological studies

Women who use oral contraceptives with a concentration of more than 35 μg/kg bw/day have a higher risk of breast cancer than women who do not use the pill. The relative risks are 1.99 and 1.27 respectively. The relation is more pronounced in women younger than 35 years. For them, the relative risks are 3.62 and 1.91 respectively (Althuis *et al.,* 2003). Epidemiological studies have shown that oral contraceptives have a protecting effect against cancer of the ovaries. Each use of OCs reduces the risk of ovarian cancer by 40 to 50 % compared to non-pill users. The reduction in the risk increases with duration of the use and remains for 10 to 15 years.

7. Risk assessment for tributyltin compounds

7.1. Hazard identification

Tributyltin (TBT) compounds are mainly used as additive in protection paints to prevent the growth of marine organisms on fishing nets and ships. They are inhibitors of the oxidative phosphorylation in mitochondria. They are responsible for the inhibition of energy transfers and result in the disturbance of energy (ATP) production, the swelling and collapsing of mitochondrial membranes and changes in the transport of ions between the lipid membranes. The influence on P450 mono oxygenase disrupts the hormonal balance and has a negative influence on respiration, growth, immune system and reproduction (Langston, 1996).

7.2. Exposure assessment

7.2.1. Dose-response relations in animals

Table 34 provides an overview of the most important effects induced by TBT in animals. For each effect, the NOAEL and LOAEL values are indicated in as much as they have been published.

Table 34. TBT: Dose-response relations in animal studies. NOAEL and LOAEL values are expressed as mg/kg bw/day, unless otherwise mentioned

Effect	Animal species	NOAEL *	LOAEL *	Organ/type of effect
Reproduction	Mouse[a]	-	5	Decreased number of pregnancies
	Rat[b]	-	5	Abnormal skeleton growth
	Rat [c]	5	10	Increase of postnatal deaths, decreased motoricity
	Mouse[d]	20	40	Decreased foetus weight
Immunological	Rat [e]	0.29	2.95	Decreased thymus weight
	Old rat[f]	0.25	2.5	Decreased thymus depending immunity
	Mouse[g]	-	0.1	Decreased cell immunity
	Monkey[h]	-	0.14	Decreased lymphocytes

a = Karrer et al. (1995), b = Schroeder (1981), c = Crofton et al. (1989), d = Baroncelli et al. (1990), e = Schroeder (1990), f = Vos et al. (1990), g = Bukovia et al. (1992), h = Baroncelli et al. (1990)

7.2.2. Dose-response relations in humans

No human data are available to characterise the toxicity of TBT. The quantitative assessment for humans has to be based on laboratory data for animals.

7.3. Exposure assessment

The most important human pathway of TBT intake is through seafood polluted by anti-fouling paints. The influence of TBT and its degradation products (MBT and TBT) caused by the erosion of PVC-tubes in water does not need to be taken into account since sufficient research results are currently not available.

No data are available on the concentration of TBT in drinking water. For surface water in Belgium, the following data are used: data for the port of Antwerp, data for the fishing port of Zeebrugge and data for the laguna Spuikom in Oostende. TBT is not present in agriculture soils. Concentrations in vegetables, meat and milk were therefore not considered.

Table 35 provides an overview of the EHDIs for the three different scenarios.

Table 35. TBT: Concentrations and estimated human daily intakes for three scenarios

Location of sample	Concentration in sediments (μg /kg ds)	EHDI (μg/kg bw/day)
Port of Antwerp	51 800	15.800
Fishing port of Zeebrugge	400	0.122
Spuikom of Oostende	30	0.009

7.4. Risk characterisation

Based on the comparison of the extrapolated values (RfD) to the EHDIs, effects are only expected to occur at the highest concentrations of TBT found in the port of Antwerp. This daily intake will be related to a decrease of cell immunity (Table 36).

Table 36. TBT: Calculation of RfD and comparison with the $EHDI_{tot}$, expressed as μg/kg bw/day. The shadowed part of the table highlights scenario values that exceed the corresponding RfD (EHDI > RfD).

Effect	LOAEL	RfD	Minimum scenario	Average scenario	Worst-case scenario
Decreased number of pregnancies	5000[a]	55.556	0.009	0.122	15.800
Abnormal skeleton growth	5000[b]	55.556	0.009	0.122	15.800
Increased number of postnatal deaths	10000[c]	111.111	0.009	0.122	15.800
Decreased foetus weight	40000[d]	444.444	0.009	0.122	15.800
Decreased thymus weight	2950[e]	32.778	0.009	0.122	15.800
Decreased immunity	140[h]	1.556	0.009	0.122	15.800

a = Karrer et al. (1995), b = Schroeder (1981), c = Crofton et al. (1989), d = Baroncelli et al. (1990), e = Schroeder (1990), f = Vos et al. (1990), g = Bukovia et al. (1992), h = Baroncelli et al. (1990).

The concentrations found in the surface water in Flanders are higher than the values found in England and Asia, especially for concentrations in the port of Antwerp. This coincides with the worst-case scenario. Antwerp is one of the largest ports in the world and is the most polluted with TBT because of intensive shipping and the cleaning of ships in dry-dock. People are not allowed to fish in the port waters, but there is a real risk of bioaccumulation of TBT in fish species that pass through the port and then migrate to areas where fishing does occur.

8. Discussions

8.1. Lack of data

The reliability of the data and the quantitative aspects of the effects hinder a complete risk analysis. Specific aspects include unknown exposure periods, absence of control groups, no dose-response data or an insufficient number of animals used in the experimental study. Few NOAEL and LOAEL values can therefore be considered reliable data. Epidemiological data are scarce: human NOAEL and LOAEL values were found for none of the five substances. Only the effects of dioxins are reported extensively.

Exposure values (concentrations in air, food, drinking water) used in calculations for the Flemish situation were - whenever possible - compared to the literature data for other countries (dioxins: Degen *et al.,* 2002; INSERM, 2000; Leonards *et al.,* 2000; Liem *et al.,* 1997; MIRA-T, 2001; atrazine: EPA, 2002; Wenzel *et al.,* 1998; BPA: Watson, 2001; EE_2: Christensen, 1998). All values are of the same order of magnitude. Therefore, major under- or overestimations in this risk assessment are unlikely.

The results often reflect incomplete data because substances are not measured in particular environmental compartments. To overcome this lack of measured data, the model of the TGD is used. Furthermore, the data used are not always representative for the Belgian population. This problem is partially dealt with by the use of the three scenarios, which determined a range of exposure concentrations within which the EHDI was estimated.

8.2. Deterministic model versus probabilistic model

A deterministic model rather than a probabilistic model is used to calculate the risk of health effects. It describes the situation and the results as a straight forward

outcome without variations. Threshold values for effects (based on LOAELs) are collected, and as soon as these values are exceeded by the estimated daily intake, a potential risk for the population is assumed. A probabilistic model would produce a more realistic image of the real exposure using an interval of concentrations to which the Flemish population is exposed (average, median, standard deviation). The latter requires far more data and is much more labour-intensive. For PCDD, PCDF and dioxin-like PCB contaminants, probabilistic intake data exist for the Flemish population, which suggest lower intakes than the EHDI levels resulting from the risk characterisation in this study. Vrijens *et al.* (2002) reported that 85 % of Flemish adolescents were exposed to less than 4 pg TEQ/kg bw/day dioxins (versus 5.79 and 14.81 pg TEQ/kg bw/day as average and worst-case, assessed in this study).

Age, environment, life style and gender were not taken into consideration in this study. The assessment is based on a simplified estimate of the human daily intake for a general population and does not take into account sensitive groups, like the elderly, the sick, the disabled, babies and children, or professionally exposed people such as fishermen or farmers who consume their own products, nor the possible cumulative effects of the different substances a person is exposed to. However, the estimated daily intake is dependent on daily intake and body weight. Hence, people with a lower body weight are assumed to have a higher risk of adverse health effects, as a result of the higher intake concentration of the toxic substance.

Nevertheless, the TGD-model is an accessible and reliable method to estimate the potential health risks of a substance for the human population. It can be used as a rapid way to determine risk.

9. Conclusions

The risk assessments are performed to determine potential risks of endocrine disrupting chemicals for the Flemish population. Except for dioxins, data for all other toxicants are characterised by insufficient information about environmental concentrations. To deal with this lack of information, data from abroad were used. Therefore, these risk assessments provide a first indication and do not correspond to definitive exposure risks for the Flemish population. It is important to monitor EDCs in the different environmental compartments in Flanders and to do so in a systemic way. Moreover, further research on the effects of the pollutants is needed.

This study has pointed to possible (exposure) problems for the Belgian population with relation to dioxins, atrazine, bisphenol A, ethynylestradiol and tributyltin

compounds. Although many uncertainties exist, results are consistent with similar findings in other countries.

Dioxins are a cause of concern: minimum exposure in Flanders coincides with the risks of decreasing numbers of sperm cells and the development of endometriosis. Immune suppression is also a risk related to dioxin exposure. A lifetime exposure to 2.56 pg TEQ/kg bw/day (minimum scenario) is also related to an increased risk of cancer.

Atrazine is not likely to cause adverse effects at the estimated concentrations. The data available and the information about well-established dose-effect relations are insufficient for a reliable application of the TGD procedure.

Exposure to an average concentration of 1.07 μg/kg bw/day of bisphenol A is associated with a higher risk of decreased sperm production, precocious puberty, changes in reproductive functions and infertility. Further research on BPA is therefore important.

An EHDI of 0.62 ng/kg bw/day EE_2 (average scenario) can be related to decreased fertility. The daily intake of women who take contraceptives containing 35 μg EE or more exceeds the RfD for some of the described effects much more than in women who do not take contraceptives. The occurrence of these effects is therefore higher among the 35 μg EE users.

Finally, the exposure to TBT is only in the worst-case scenario (Port of Antwerp) associated with decreased cell immunity.

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PROBLEMS IN ASSESSING LOW DOSE EFFECTS OF ENDOCRINE DISRUPTERS

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Summary

Though endocrine disrupters and their effects in wildlife and human health have been the objects of intensive scientific research for almost fifteen years, a conclusive concept for risk assessment and risk reduction of this group of chemicals is still lacking. In many cases non-linear or biphasic dose response relationships are observed when endocrine disrupters are tested in toxicology and ecotoxicology. This means that effects of low doses of these chemicals cannot be predicted from experiments with high doses and thus the central paradigm of regulatory toxicology becomes questionable. As the risk assessment of endocrine disrupters remains to be a field characterised by a high degree of uncertainty. Political bodies like the European Parliament request immediate regulation on the basis of the precautionary principle.

1. Introduction

Chemicals with endocrine disrupting properties have been a subject of concern for scientists, politicians, and the general public for the last fifteen years. Reviewing this time period, public, political and scientific interest has focused on two issues:

The first one is a concern about decreasing human reproductive health. The most prominent secular trends in male reproductive health such as decreasing sperm quality (Swan *et al.,* 2000), increase in male congenital malformations (Toppari, 2002) and a growing incidence of testicular cancer in most industrialised countries

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 283–296.

(Toppari *et al.,* 1995) have attracted much public interest and have triggered extensive press coverage of the issue of endocrine disrupters. Interestingly potential effects on male reproductive health by endocrine disrupters have been in the public eye much more than effects on female health though a large number of papers indicate that endocrine disruption may affect endpoints of female reproductive health as much as or even more than male ones (Damstra *et al.,* 2002).

The second source of concern is endocrine disrupter caused effects in wildlife such as imposex in prosobranch snails, feminisation in fish and congenital malformations in reptiles (Gies, 2003). Effects in aquatic animal species, in particular, have been regarded as early indicators or warning signs for human risk and their role as sentinels for potential effects on the human foetus has recently been discussed (Sharpe and Irvine, 2004).

2. The human-wildlife connection

It is remarkable that in the field of endocrine disruption, effects on humans and on wildlife have been discussed in close connection. For example wildlife effects have frequently been regarded as indicators of possible similar effects in humans. On the other hand disruption of the hormonal system in wildlife populations can serve as a marker of exposure (Hecker *et al.,* 2002). For example vitellogenesis in male fish, as often seen in many surface waters worldwide indicates that the concentration of estrogenic substances in a river or lake is high enough to cause biological effects. These biological markers of exposure have some advantages over direct measurement of endocrine disrupting substances. Biological markers mirror the total activity of all natural and synthetic substances that are relevant as modulators of the hormonal control of the endpoint under study. Moreover, they are able to integrate effects over a long time period. Thus, for example, vitellogenin levels in fish will be elevated even if the estrogenic chemical stimulus is present only for a limited time and thus might be missed by chemical water analyses.

The interdependencies between toxicological and ecotoxicological aspects, particularly in endocrine disruption, have given rise to the development of a concept of integrated risk assessment, describing risks for human health and ecological systems in an integrated manner (IPCS, 2001). The main reason behind this approach is that it improves the quality and efficiency of assessments through the exchange of information between human health and environmental risk assessors and the coherence of inputs to the decision-making process. With respect to the latter, human health and ecological risk assessors have traditionally been two

different subgroups of scientists often communicating poorly and providing decision makers with inconsistent input that results in contradictory impressions of the nature of risks. This is due to differences in approach, and the integrated approach seeks to eliminate these inconsistencies. A working group advising the scientific committees of the EU has comprehensively reviewed current European risk assessment procedures in toxicology (European Commission, 2000). This working group came to the conclusion that progressive harmonisation of human health and environmental risk assessment procedures within the EU is both of practical importance and scientifically sound. However, it was recognised that full harmonisation of risk assessment procedures in the EU is not achievable in the short to medium term.

Both toxicological and ecotoxicological assessment of endocrine disrupting chemicals require a special view as these chemicals have a number of characteristics that have not commonly been considered in traditional risk assessment procedures. These unique features are:

- There are windows of increased sensitivity mainly during the early postnatal or prenatal development where these substances act in low concentrations or show different effects from those in later life stages.

- The effects may become observable at a later life stage, when actual exposure may have ceased. This is not only true of diseases that are known to have a very long latency time like cancer. Exposure to chemicals adversely affecting male reproductive health may occur prenatally and effects such as lower sperm counts may become evident decades later when the boys reach puberty.

- Short exposure times during critical windows of susceptibility to that chemical may lead to life-long irreversible effects.

- High and low doses of endocrine disrupting chemicals may cause different and even opposite effects. In regulatory terms this means that effects of these chemicals at low doses cannot be extrapolated from the results of high-dose experiments.

3. Low dose effects

Commonly two definitions of low dose effects are used in the literature. These definitions served as the basis for the German Low-Dose Workshop in 2000 (Chahoud *et al.,* 2001) and for the US National Toxicology Program's endocrine disrupters peer review (Melnick *et al.,* 2002). Those effects were regarded as low

dose effects that occur either at concentration or dose levels to which humans and wildlife are generally exposed or effects occurring below the Lowest Observed Adverse Effect Level (LOAEL) of traditional (eco) toxicological assessment. The Bisphenol A panel of the US NTP Low Dose Peer Review for example chose a dose factor of 10 lower than the LOAEL for oral exposure established by the US EPA.

These definitions clearly show that low dose effects are not by their nature a unique biological phenomenon but rather a problem in chemicals assessment as adequate models to predict these effects are lacking at present. The regulatory thresholds or the environmental concentrations of a substance define a low dose effect rather than a biological mechanism. The discussion on low dose effects reflects inadequate toxicological testing by the traditional testing regimes for regulatory purposes.

Even cost intensive multi-generation tests do not routinely include subtle and transgenerational endpoints such as sperm quality in the progeny of dosed animals, slight but functionally important changes in prostate size, morphology or histology endpoints. So they can hardly serve as models for changes caused by endocrine disrupters and effects epidemiological studies postulate are caused by this group of substances.

Another difficulty in assessing low dose effects is that in many cases non-linear or biphasic dose response relationships are observed. These observations are obviously independent of the system under study as they occur in mice with prostate size as an endpoint (vom Saal *et al.,* 1997) as well as in snails in regard to clutch size (Schulte-Oehlmann *et al.,* 2001). The IPCS global assessment of endocrine disrupting chemicals (Damstra *et al.,* 2002) points out that endocrine disrupters often act by mimicking or antagonizing the actions of naturally occurring hormones. These hormones are already present in the body at physiologically functional concentrations. As a consequence their dose response-relationships differ from those of other environmentally relevant chemicals. No common dose response relationship for all effects and all endocrine disrupting mechanisms should be expected. This means that traditional toxicology is in a dilemma in the field of endocrine disruption. The central paradigm, that effects at low doses can be extrapolated from high-dose effects (or, in other words: if high concentrations of a chemical cause no harm, then low concentrations won't either) may not hold true. As almost all risk assessments of endocrine disrupting chemicals are made on the basis of this paradigm, the results of these assessments have to be regarded as questionable.

3.1. Low dose effects of bisphenol A

Bisphenol A has become arguably the best and most intensively researched synthetic substance model for endocrine disrupters. Nearly every day in the year an article about Bisphenol A is published and every fourth day an article about hormonal aspects of this substance appears (Figure 16). Bisphenol A is a monomer for the production of polycarbonates and epoxy resins. Approximately 700,000 tons of Bisphenol A are produced and used within the European Union (Joint Research Centre, 2003). Because of its intensive use the substance is found in many environmental compartments; concentrations in surface waters are typically between 10 and 100 ng/L.

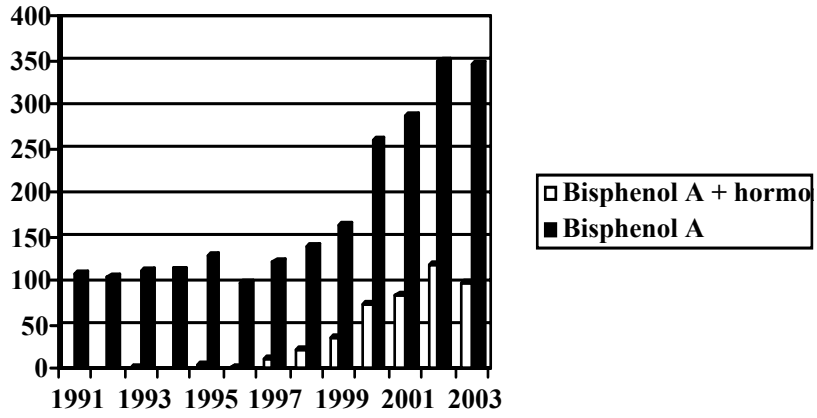

Figure 16. Number of papers cited in Medline on Bisphenol A and on hormonal aspects of Bisphenol A from 1991-2003

The issue of low dose effects of Bisphenol A remains controversial. As a matter of fact results of low dose tests did not find their way into the assessments of regulatory bodies worldwide. Table 37 gives a summary of studies in mammalia with effect levels below the NOAEL, which the European Scientific Committee on Food (SCF) currently sets at 5 mg/kg bw/day. Applying a safety factor of 500, this gives a temporary TDI of 0.01 mg/kg bw (European Commission, 2002). For this TDI derivation, the SCF regarded the findings in the low dose studies as not being robust enough for derivation of a TDI, stating that uncertainties in the database remain. To respond to these concerns the SCF introduced an extra safety factor of 5, maybe as a tribute to the precautionary principle. A recent review (vom Saal and Hughes, 2005) found among 115 published *in vivo* studies on Bisphenol A 94 papers reporting significant effects. 31 papers out of them reported effects below **50 µg/kg bw/day Bisphenol A either in vertebrates or in invertebrates.**

Table 37. Summary of mammalian studies on bisphenol A with effect levels below 5mg/kg bw/day, oral administration

Dose [µg/kg bw/d]	Organism, age at dosing	Effect	Reference
0.7	Rat, 3 generation study	Paired ovary weight in F2 generation⇓, uterine weight in F0 ⇓, anogenital distance in female F2⇓	Tyl *et al.* (2002) (Effects were not regarded as relevant by the authors)
2	Mouse, gestation day 11-17, offspring	Aggression ⇑, Testis weight ⇓ in offspring	Kawai *et al.* (2003)
2	Mouse, gestation day 11-17, offspring	Prostate weight ⇑, epididymis weight ⇓	Vom Saal *et al.* (1998)
2	Gerbil, females, 3 weeks after pairing	Changed maternal behaviour	Razzoli *et al.* (2005)
2	Rat, 3-generation	Spermatogenesis, sperm quality	Peknicova *et al.* (2002)
2.4	Mouse, gestation day 11-17,	Vaginal opening, first oestrus in offspring	Howdeshell *et al.* (1999)
2.5	Mouse, 5 wk	Immune, IFN-gamma and IgG2a ⇓	Sawai *et al.* (2003)
10	Mouse, gestation day 14-18	Maternal behaviour in offspring	Palanza *et al.* (2002)
10	Mouse, gestation day 14-18	Number and size of dorsolateral prostate ducts in offspring ⇑	Timms *et al.* (2005)
10	Mouse, gestation day 11-18	Long-term alteration in neurobehavioral functions in females	Laviola *et al.* (2005)
20	Mouse	Chromosomal aberrations, aneuploidy ⇑	Hunt *et al.* (2003)
20	Rat, 13 wk	spermatogenesis⇓	Sakaue *et al.* (2001)
20	Rat, pregnancy	Vaginal morphology in offspring	Schönfelder *et al.* (2002)
20	Rat, day 1-21 postnatal	Changes in ultrastructure of uterus and vagina	Fukumori *et al.* (2001, abstract)
25	Mice, gestation day 8-23,	Structural and histological changes of prostate in offspring	Ramos *et al.* (2001)

Table 37. Continued

Dose [µg/kg bw/d]	Organism, age at dosing	Effect	Reference
40	Rat, gestation day 14 – postnatal day 6	Sex-associated behavioural changes in offspring	Dessí-Fulgheri *et al.* (2002)
40	Rat, pregnancy and lactation	Aggression behaviour in offspring	Farabollini *et al.* (2002)
40	Rat, pregnancy and lactation	Changes in spontaneous and amphetamine induced behaviour in offspring	Adriani *et al.* (2003)
40	Rat, pregnancy and lactation	Decrease of playful interactions in offspring	Porrini *et al.* (2005)
40	Rat, pregnancy and lactation	Changes in maternal behaviour in adult females	Della Seta *et al.* (2005)
50	Mouse, gestation day 16-18	Anogenital distance and prostate size ⇑, epididymal weight⇓ in offspring	Gupta (2000)
100	Rat, juvenile	Breast tissue maturation⇑	Colerangle and Roy. (1997)
100	Rat, day 6 of pregnancy to lactation	Body weight in offspring⇑	Rubin *et al.* (2001)
100	Rat, days 1-14 postnatal	Sperm content in testis and epididymis ⇓	Bowers *et al.* (2001, abstract)
100	Rat, pregnancy	Sex differences in brain development in offspring	Funabashi *et al.* (2004)
100	Rat, gestation day 3 to postnatal day 20	Reception of fear provoking stimuli in male offspring	Negishi *et al.* (2004)

4. Political demands for the assessment and regulation of endocrine disrupters in Europe

The European Parliament, the European Commission and many national governments in the EU have come to recognise the importance of the issue of endocrine disrupters. They have been asking for greater activity in the fields of research, more rapid risk assessment within the chemicals safety programme and subsequent risk reduction measures.

It was shortly after a series of scientific congresses in Europe in 1995 and broad media coverage induced by them that the European Parliament took up this issue. In 1998 the Parliament adopted a resolution on this class of chemicals (European Parliament, 1998), which broadly covered the issues of assessment, regulation, and research. In this document the European Commission was urged to take up this issue and to develop a regulatory strategy for this class of chemicals, and to use all available instruments such as the European chemicals legislation and the Water Framework Directive to control endocrine disrupters. In 2000 the European Parliament adopted Report A5-0197/2000 on endocrine disrupters (European Parliament, 2000), emphasising the application of the precautionary principle and calling on the Commission to identify substances for immediate action. The MPs asked, in particular, for the identification of endocrine disrupters for immediate action without further tests, a European screening and testing strategy, and a legal framework for endocrine disrupters based on the precautionary principle and shifting the burden of proof to the producer. Again the Parliament pressed for intense research in this field, placing particular emphasis on low dose effects. The Parliament evidently had the impression that among European risk assessment procedures no procedure was available to take account of this phenomenon. With the statement that it "… finds that the strategy must recognise that several endocrine disrupters (EDs) have now been found to cause effect at a very low dose level and that there is uncertainty about effects of a mixture of EDs and synergetic effects;" the European parliament very frankly expressed that current assessment procedures are not very well in line with its understanding of precaution in chemicals risk assessment.

The European Commission responded to these requests in 1999 with a communication on a community strategy for endocrine disrupters (COM 99/706) (Commission of the European Communities, 1999), which sets out actions that have to be taken in the short, medium, and long term. The most important short term actions proposed were; prioritising the various substances, primarily with respect to the risks associated with their hormonal effects; applying existing legislative instruments; and measures in respect of monitoring programmes, international co-ordination and public information. For the medium term the Commission proposed systematic assessment of endocrine disrupters and further development of test methods and research. In the long term, the EU legal framework on chemicals, crop protection agents and biocides is to be adapted, prioritising the various substances, primarily with respect to the risks associated with their hormonal effects, applying existing legal regulations (like the Existing Substances Regulation (EC 793/93)) and deciding on monitoring programmes, international co-ordination and information for the general public. In a second communication, from 2001, on the implementation of

the strategy (Commission of the European Communities, 2001a) the Commission described the progress made during the preceding two years in implementing a research programme, supporting the OECD strategy to develop test guidelines for endocrine effects and incorporating risk assessment and risk regulation procedures for hormonally active substances into existing EU legislation in the areas of chemicals control and protection of environmental media.

The OSPAR Convention (Convention for the Protection of the Marine Environment of the North East Atlantic) also requires further action. In the framework of this 1992 Convention, pollution of the North Sea by hazardous substances must be reduced to harmless levels by the year 2020, the final aim being the total elimination of these substances within one generation. In the OSPAR framework chemicals with an effect on the endocrine system are explicitly covered by the definition of hazardous substances (OSPAR, 1998). OSPAR has prepared a list of substances of possible concern, from which a list of substances for priority action was derived. Both of these lists contain a number of endocrine disrupting substances such as phthalate esters and tetrabromobisphenol A. One of the most challenging tasks for the European Commission is to provide the legal instrument that will enable national governments and the European Union to fulfil the commitments they made in the OSPAR Convention.

Regarding legislative action, the European Commission plans to incorporate specific provisions on endocrine disrupting substances into three sections of European legislation, in particular.

The revised General Product Safety Directive that came into force in early 2004 does not, contrary to previous Commission plans, explicitly address endocrine disrupters. For the protection of consumers from such substances it may be important that the directive includes *inter alia* a simplification of conditions and procedures for urgent measures at Community level. This may facilitate direct actions to stop the marketing of products that may pose a risk to consumers in general or to specifically vulnerable consumer groups. High levels of phthalate esters in toys for small children is a recent example where the Commission took immediate action to suspend marketing until uncertainties in risk assessment and regarding the feasibility of risk reduction measures are finally clarified.

In addition, the issue of endocrine disrupters is addressed specifically in the context of new (the Water Framework Directive) and existing legislation, and in the White Paper on a strategy for a future chemicals policy.

For example, the authorisation provisions under the proposed new system for chemicals assessment and regulation (REACh) (Commission of the European Communities, 2003) will ensure that risks from the use of substances with properties of very high concern are either adequately controlled or that these substances are authorised on socioeconomic grounds. These decisions will take into account all available information on alternative substances or processes, in which case the authorisations will normally be time-limited. Substances of very high concern are defined as: substances that are category 1 and 2 carcinogens or mutagens; substances that are toxic to the reproductive system of category 1 and 2; substances that are persistent, bioaccumulative and toxic or very persistent and very bioaccumulative; and substances such as endocrine disrupters which are demonstrated to be of equivalent concern.

The White Paper of the Commission on the future chemicals policy (Commission of the European Communities, 2001b) mentioned low dose effects as a research issue, and not, as the European Parliament had requested, as a task for further development of risk assessment and risk reduction for endocrine disrupters:

"Particular research efforts need to be made to assess the potential adverse effects of chemicals on endocrine systems of humans and animals. Research on endocrine disrupters is also – among others - addressing the effect of low doses, long term exposure and exposure to mixtures of chemicals, and the impact of the endocrine alterations on carcinogenesis.". In the recent proposal of the Commission for a new chemicals policy (REACh) it remains unclear whether endocrine disrupters will be regarded as a class of chemicals in need of special attention and an assessment procedure that takes possible low dose effects into particular account.

5. Conclusions

The risk assessment of endocrine disrupters remains to be a field associated with high uncertainty even after fifteen years of intensive research. Low dose effects, which are observed quite frequently within this class of chemicals, challenge central paradigms of toxicology. For endocrine disrupters it is probably not possible to extrapolate from high doses usually given to animals in toxicological experiments to low doses, which are environmentally relevant. Regulatory bodies frequently dismiss experiments showing low dose effects. This does not solve the problem of modern regulatory toxicology that a totally new approach for the assessment of dose-response relationships is needed.

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GETTING THE MESSAGE ACROSS – WWF'S AND ISDE'S PERSPECTIVES ON COMMUNICATION STRATEGIES TO REDUCE EXPOSURES TO HAZARDOUS CHEMICALS

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Summary

This paper addresses the topic of communicating the issue of hazardous chemicals to different audiences with regard to the ultimate goal of reducing the impact of chemicals on health and the environment. It is a broad topic, because action to reduce risk can be taken at many levels, ranging from the level of the individual right through to regulatory action to ban an individual chemical under a global Convention.

Non-Governmental Organisations (NGOs), such as the International Society of Doctors for the Environment (ISDE) and the World Wildlife Fund for Nature (WWF), the global environment network, carefully plan their communication strategies to embrace a mix of target audiences, with a view to achieving the greatest protection of humans and/or the environment with their relatively scarce resources. This paper summarises some of the strategies adopted by WWF and ISDE, and considers more generally some of the questions that need to be addressed prior to embarking on a campaign.

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 297–310.

Organisations also need to consider what their particular niche is, in terms of what they can do best, and where they will have most added-value. Vehicles for communication are numerous and include, amongst others, conferences, websites, leaflets, research journals and other publications. For some audiences, well-founded scientific arguments will be most persuasive, but other audiences may have a more emotional basis for determining what is acceptable. It therefore needs to be recognised that a mixture of different approaches will be required for communicating with different audiences, and also, to achieve the optimum results the approach may need to change over time. Furthermore, working with other groups and sharing experiences and knowledge can undoubtedly improve effectiveness.

1. Introduction

In getting the message across, health professionals and environmental NGOs can play a useful role, as both groups are respected and trusted by the general public. For example, according to a recent survey in Britain, the public trusts environmental groups to tell the truth about the risks from the chemicals used in household goods, over and above Government, Government agencies, chemical manufacturers, industry or government scientists, or even university scientists (Scientific Alliance, 2003).

It is now an accepted fact that for most chemicals currently on the market there is insufficient toxicity information publicly available to undertake even a rudimentary risk assessment. Studies on both sides of the Atlantic have revealed a paucity of available toxicity data (Allanou *et al.,* 1999; US EPA, 1998; Goldman and Koduru, 2000). Furthermore, more and more studies now suggest that exposure to chemicals can play a role in many diseases and disorders including birth defects, cancers, infertility, and deficits in immune function and brain function (for review see IPCS, 2002). Concern about the effects of chemicals on health is therefore now justifiably widespread. Indeed, a recent opinion poll commissioned by the Directorate General Health and Consumer Protection, showed that a staggering 93% of Europeans believe that chemicals negatively affect health (Eurobarometer, 2003). The need to protect humans and wildlife from adverse effects caused or exacerbated by chemicals is thus an important challenge, and communication will play a central role in delivering a better level of protection.

2. Disseminating the message: Issues to consider

In planning strategies to disseminate the message, three initial questions need to be addressed. These include:

- what do you wish to achieve?
- to whom do you want to communicate?
- what is your message?

Subsequent questions revolve around finding the right niche and working areas that complement activities that are already on-going. Duplication is wasteful, but groups may be able to augment each other, or repeat successful strategies in other countries.

The desired outcome can range from changing life styles to changing legislation, but it might be assumed that only the latter results in gains for the wider population, because focussing communication on encouraging the public to change life styles is likely to benefit just a sector of the population who are well-informed and risk-averse. However, if public attitudes can be changed, in time this sort of behaviour can become normal, which in turn has a big effect on policy-making which is largely based on the 'accepted-norms.' Similarly, consumer demand can affect retailer behaviour, which can in turn guide manufacturers to produce safer chemicals.

Changes in legislation take years to effect, yet meanwhile many people appear to be increasingly keen on obtaining information on how better to protect themselves. It should also be recognised that informing the public, can help them be active participants in the push to tighten legislation. Furthermore, even with good regulation of chemicals, there will still be a need to inform consumers about correct use.

With regard to reproductive health, target audiences may include the general public, young adults prior to trying for family, infertile couples, pregnant women, health professionals, and the media, industry, or policy makers. This paper will cover issues related to communicating with the following target audiences: the general public; policy makers; health professionals; and industry.

The message needs to be tailored for different audiences, but still be in line with achieving the end goal that you have selected. For example, WWF's goal is to end the chemical threat to biodiversity. Therefore, getting better controls over chemicals that can impact fertility is crucial. One of the principal ways WWF seeks to do this by trying to change European legislation, and that has been one of WWF's main

focuses of activity for several years. In the late 1990s, Governments in the EU woke up to the realisation that the current lack of regulation of industrial chemicals was untenable, so since that time much of WWF's work on toxics has been focussed on communicating with policy-makers on the details of what new legislation is needed. During the early days WWF was one of the few NGOs active in this area. WWF has also worked to achieve an EDC strategy in the EU, and was represented on the EU EDC sub-group on Integrated Monitoring of Endocrine Disrupters set up under the EU Environment and Health Strategy. This illustrates that WWF seeks to communicate its objectives at all levels, including technical input in legislative and scientific fora, as well as more generally to the wider public.

With the proposed REACh legislation, we now have a once-in-a-lifetime opportunity in the EU to get better controls over industrial chemicals. Knowledge about the types of chemicals that have caused long-term damage to wildlife in the past, leads WWF and other NGOs to want exposures to certain chemicals eliminated. These include chemicals that are very persistent and very bioaccumulating (vPvB chemicals); those that are persistent bioaccumulative and toxic (PBTs); and chemicals such as those with endocrine disrupting (ED) properties or those that can subtly derail biochemical signalling pathways. Bioaccumulating chemicals pose a threat to future generations because they can be passed on in the womb, or via the egg, to cause effects in offspring at doses that would not cause effects in adults. WWF's goal is to ensure that these chemicals are only used when there is an over-riding societal need, no safer alternatives, and measures to minimise exposures are in place. This means that we are trying to achieve legislation, which delivers the phasing out of these chemicals of very high concern if there is a safer alternative available. The text of the new proposed EU legislation does not yet achieve this, although these chemicals will require prior authorisation and certain conditions will need to be fulfilled before authorisations for use are granted.

The proposals for the REACh legislation have been refined over time, and were first laid down in the White Paper of February 2001 (CEC, 2001), with the current draft regulation being released in October 2003. Over time, the opposition to this legislation has grown, not only from industry within the EU, but also from countries outside the EU, particularly the USA (US House of Representatives, 2004). As this opposition has grown, WWF has re-assessed its tactics, and now, rather than just concentrating on communicating with policy makers, it has recognised that it is important to engage the public, in order to help them to participate in the decision-making process. The weight of public concern needs to be felt, in order to counteract the powerful forces of industry that have fought to water-down the legislation.

After sounding out focus groups made up of WWF members, it was apparent that some WWF supporters were rather perplexed about why WWF was working on chemicals in household products. It was not an obvious organisational niche that would easily enhance the 'brand'. Therefore, WWF needs to keep enforcing its central message that work on species and habitat conservation will come to nothing unless we tackle this toxic threat to wildlife reproduction.

3. Communicating with the public

To be successful in changing the public, you need to ask first not only what you want to change and who you want to influence, but perhaps most importantly what makes your target audience 'tick'. Understanding the value modes of the people you want to change can help frame how you communicate with them, and provide the key to changing their behaviour.

For other groups that are trying to disseminate information, public outreach might be targeted at getting the public to change their life style in order to reduce their risk. In contrast, WWF primarily wants to empower the public as a force for regulatory change, but recognises that alongside this there is a duty to provide information about what people can personally do to reduce their risks. Also, focussing on personal risk and life style is a way of drawing people in, and then if the public so desire, they can take steps to lobby for better legislation.

Whatever the aim of communicating with the public is, one must be aware that the public has a wide range of understanding. Many people are ignorant about the extent of chemical contamination and the potential risks, and care must be taken not to reach the wrong outcome. For example, WWF made a clear decision not to sample breast milk for fear of driving mothers away from breast-feeding. The WWF strategy was not only aimed at informing people, but also to provide information to enable them to act on this concern at both a personal and a political level.

As an example of WWF's tactics, in May 2003 WWF UK launched a campaign to involve the public in the debate on chemicals. Recognising the need for more resources, including people's power, WWF UK joined forces with The Co-operative Bank and the Women's Institute. In 2003, their contamination campaign began and sought to draw attention to the fact that humans were now carrying a body burden of numerous pollutants. In showing the on-going presence of PBT chemicals, WWF aimed at garnering widespread support for eliminating current use of chemicals with similar characteristics. A biomonitoring tour was undertaken, and 155 selected people and WI members from 13 locations in the UK volunteered to have their

blood tested for 77 chemicals, including 45 polychlorinated biphenyls (PCBs), 12 organochlorines, and 21 polybrominated diphenyl ethers (PBDEs). Some members of the public were shocked to find that they had so many persistent chemicals in their blood (see WWF UK, 2003). Following on from this, WWF's campaign went international, and subsequently blood was taken in the Brussels parliament from 47 men and women volunteers of all ages from 17 countries in Europe, including 39 MEPs, and some observers from Accession Countries. This time more chemicals were monitored, and the list included phthalates and some perfluorinated coupounds. Extensive media coverage was ensured as some representatives of the Press were also sampled, as well as people such as the then UK Environment Minister, Michael Meacher, and the then Commissioner for DG Environment, Margot Wallstrom. TV and press interest has resulted in widespread dissemination of the message that we are all contaminated with many persistent and bioaccumulating chemicals, and that there was something that could be done about it (WWF, 2004).

WWF then harnessed some of the concern felt by these people and directed it towards their Members of the European Parliament (MEPs), who will ultimately vote on the proposed REACh legislation. Women volunteers from the UK biomonitoring survey, travelled to the European Parliament to demand safer chemicals in everyday products. They met with MEPs and were certainly a force to be reckoned with. One Conservative MEP stated that he would now have to look at the issue with a 'fresh perspective.' A 77,000 strong petition was delivered to the President of the European Parliament Petitions Committee, calling for an end to the chemical contamination of people and wildlife (WWF, 2004).

Extensive media coverage and outreach is still underway, not only based on the blood sampling initiative in the EU, but also based on the blood sampling undertaken by WWF at the June 2004 Fourth Ministerial Conference on Environment and Health in Budapest.

Biomonitoring is a powerful tool, but it should be used sparingly, and for maximum value should be linked to a definite policy. Harnessing people's concern, and getting them to write to politicians, or lobby decision makers, or to write in their local newspaper, can help the public to take an active role in democracy and lend their weight to the decision-making process.

At a personal level, life style choices to reduce exposure, such as eating more organic food, and reducing exposure to certain chemicals in food, packagings, and cosmetics, are all options about which people want information. For many groups

that are trying to disseminate information, public outreach might be focussed on getting the public to change their life style in order to reduce their risk, or the risks to their children, for example, by not smoking at home.

Tools to disseminate information include environmental education in schools, meetings and conferences, and computer web sites and leaflets, but it is always a challenge to package what is often complex information into something that is easily understood, without compromising its factual content. In addition to web sites run by NGOs, there are also official web sites run by Government agencies in some Member States, for example, which provide advice on which fish to avoid in order reducing mercury intakes for pregnant women (FSA, 2005). However, it seems that there is a lack of official "one-stop-shops" where all advice for pregnant women is collated and available. Moreover, it is likely that Government agencies will be loathe to provide information on ways consumers can avoid contact with certain chemicals, because such advice might be tantamount to admitting a lack of adequate regulatory control.

4. Communicating with policy makers

With national members spanning the globe, each national chapter of ISDE, or national office of the WWF, is able to engage National Ministries and Agencies for Environment and Health. This is critical due to the fact that historically, most public and private sector organizations are structured with two separate programs for addressing environment and health matters. The European Union took a bold step in addressing this historical divide through the EU Ministerial Conference on Environment and Health to be held in Budapest in June 2004, which for the first time brought together the policy makers and regulators within whose domain the linkages between human health and the environment reside.

Traditionally, Environment Agencies have been the most advanced in understanding the linkages between human health and the environment. In fact, many of the discharge and emissions standards in environmental regulations are based on research designed to protect human health. On the contrary, Health Agencies have historically been tasked with delivering health care, with little or no emphasis on prevention to eliminate the various sources of human disease and illness.

Other government services and agencies provide opportunities for integrating the linkages between human health and the environment. The regulatory and functional domains of transport, sanitation and public works, public housing and education all possess activities that impact human health directly and indirectly. Understanding

these linkages prior to policy-making or rule-making can result in cost savings from reduced illness, accidents and health-care costs, as well as ancillary benefits such as increased worker productivity.

Accordingly, ISDE works with national policy makers and regulators, as well as International Organizations such as the World Health Organization (WHO) to deliver information, data, and training material that support the development of rational and sound health and environmental policy.

Governments often have official consultations on various legislative proposals, or parliamentary committees that scrutinise legislative proposals, and feeding in to these at an early stage is crucial. In attempting to influence policy, it is important to recognise that often policy-makers welcome help to find practical solutions as to the best way forward.

Many policy makers are not scientists, and so often one of the most valuable things that scientists can do is to help put the wealth of published research on a particular topic into something understandable and succinct. Review papers might not be the most prestigious in terms of delivering new science, but in terms of communicating where the science is up to, they are very valuable indeed.

The reality underlying certain statements can be hidden by rather technical jargon. For example, scientists well know the meaning of statements such as ‘there is no evidence that x causes such and such an effect’, and ‘there is a lack of data on the effects of x’ – but to the untrained eye the first can suggest that x is safe. There is a need for scientists to convey the reality of the situation in ways that are immediately clear to all.

Reports by august bodies can be particularly influential with policy makers as they represent a reputable and trusted source. Examples would include: the CSTEE report on endocrine disrupters (1999); the European Environment Agency’s (EEA’s) and UNEP’s report on Chemicals in the European Environment: Low Doses, High Stakes? (1998); the EEA’s Late lessons from Early Warnings: the precautionary principle 1896-2000 (2001); and the UK’s Royal Commission on Environmental Pollution’s report ‘Chemicals in Products: Safeguarding the Environment and Human Health’ (2003). However, reports by national groups tend to have less influence outside their national boundaries.

To underpin the WWF’s outreach to the public, and to demonstrate that its campaign is founded on sound science, a Scientists Declaration was drafted, and well-known

scientists from the EU were asked to sign. This Declaration has now been signed by over 60 scientists and can be found on the WWF UK's web site (http://www.wwf.org.uk/chemicals/). More signatures are welcomed. It outlines what the WWF is trying to achieve in legislative reform, and was used to put pressure on European Commission Directorates to support tighter controls on endocrine disrupting chemicals, and very persistent and very bioaccumulating chemicals.

Similarly, an effective way for scientists to communicate to policy makers may be through consensus statements, because these can instantly show that there is a good body of scientists with the same view of what the data mean. Also, consensus statements can provide a vehicle for scientists to outline not only what is known, but also what is suspected, and even what should be done. WWF has worked to try to bridge this science-policy interface. Theo Colborn, a former senior scientist with the WWF US, convened meetings to deliver consensus statements on many topics related to the effects of chemicals. These statements started with the now famous Wingspread statement of 1991, which outlined the known and predicted effects of endocrine disrupting chemicals on wildlife and humans (Colborn and Clement, 1992). This Wingspread statement, which has stood the test of time, was used by the WWF in Europe in order to help deliver an EU EDC strategy. It set out the concerns of a group of well-respected scientists under headings such as: 'we are certain of the following…'; 'we estimate with confidence…'; 'current models predict that….'; and 'our judgement is that…'. Therefore, consensus statements can provide a useful lobbying tool to influence policy-makers.

The European Commission has highlighted the need to integrate research findings and to strengthen the interface between research results and policy development (EC, 2004). Scientists can certainly play a useful role in this process.

5. Communicating with medical and health professionals

The World Health Organization (WHO) estimates that up to 25% of the global burden of human disease comes from environmental sources, with up to 47,000 persons dying each year from chemical poisoning (WHO, 2005).

As a global NGO of medical doctors, scientists and health professionals, the ISDE undertakes technical training, education, awareness-raising and research to highlight the linkages between human health and environmental sources. ISDE members consider their peers in the medical and health communities to be primary targets for capacity-building regarding these issues. This is due primarily to the fact that the

medical and health professions generally are trained to treat illness with little emphasis on prevention.

The existing opportunities to engage medical and health professionals in these issues include continuing professional education. However, given the realities associated with professional practice, there is little opportunity for a major shift toward prevention, or introducing new subject matter and training related to human health and the environment. Furthermore, statistically, most doctors receive news and information on health and treatment developments from vested interests in the health care industry, and in particular, pharmaceutical companies. Any number of reports and studies may be found to demonstrate the association between doctor education and industry, including the statistical average of $21,000 per doctor spent by Pharmaceutical products companies on marketing doctors. See, for example, the World Medical Association (www.wma.org), the Medical Journal of Australia (www.mja.com.au/index.html) or the British Medical Journal (bmj.bmjjournals.com).

At the same time, doctors and health professionals offer one of the best avenues for advocacy and reaching the public. The ISDE partners with Paediatricians to advocate for children's health and the environment. This is due primarily to the subject matter expertise possessed by Paediatricians who focus exclusively on children's health; are expert in the unique developmental, neurological and biological needs and vulnerabilities of children; and who know about the effects of toxic exposures during these developmental stages of a child.

Further, pediatricians are well-positioned for advocacy, as they represent a trusted, credible reference within society; exert influence among medical and health peers; are key partners to advocate in society and government; are adept at explaining complex matters in simple language; and, are in close contact with children and their families in the community setting.

WWF considers that health professionals have a very important role to play, both in data-gathering and in communication. Being in the front line of health care, doctors are well placed to find out what is really happening to human health, and can be instrumental in gathering data on trends in certain diseases and disorders. They are also well placed to communicate with the public, and will be a powerful force for change. However, it is clear that some clinicians know very little about the growing literature on the role of the environment in disease, and spend relatively little time engaged in disease prevention as compared to treating disease. There may be a need to educate some clinicians and involve more of the medical profession in the drive

to understand the links between environment and disease. There is also a need for the health and environmental NGOs to work together, and also to involve the health care charities, in order to create a more powerful force for health protection.

6. Communicating with industry

It should be recognised that industry has a role to play in helping steer society to a sustainable and safer way of doing business. Some chemical manufacturers may see a market advantage to finding safer alternative chemicals, or they may be keen to safeguard their reputation and good 'brand' name. Others may be stimulated by more negative concerns, and recognise that future liabilities may accrue from the continued production of dangerous chemicals. Moreover, retailers must serve the public, and so for example, Ikea has removed brominated flame retardant chemicals from its products, in part due to pressure from NGOs. Similarly, the Co-op supermarket chain in the UK announced in May 2004 that it has removed certain chemicals, such as artificial musks and phthalates, from a range of its household products. This followed the publication of an opinion poll suggesting that 6 out of 10 shoppers were ready to boycott goods that are ethically unsound (Jha, 2004).

An innovative approach to engage industry has been utilized by ISDE to reach local communities. In order to bridge the gap between the occupational and community settings, the ISDE works with various private sector organizations through Corporate Social Responsibility (CSR) initiatives. Most industry in the developed world has regulatory mandated Environment, Health and Safety (EHS) programs which have a fairly significant history of integrating the health impacts from environmental sources, such as chemical exposures. These EHS programs provide a basis through which to reach the local communities where both the current and future generations live. However, while many major industrial 'brands' have meritoriously required overseas contract manufacturers to implement EHS programs, the level of training and understanding for fulfilling these EHS requirements is rather low.

Specifically, within the developing-country context, the issue of maternal health offers a prime example for the need to address chemical exposures. Within the footwear industry, one of the largest operations in Asia employs upwards of 60,000 workers, the vast majority of whom are women of child-bearing age. While there may be a basic program of EHS standards, the understanding by the workers of the impacts of daily exposures to the mix of solvents, adhesives and other chemicals related to the manufacture and assembly of footwear, is rarely understood. This is

further compounded by the fact that the occupational setting may in fact be the less serious threat to worker's health than the local community setting, where water and sanitation issues threaten health, and in particular the potential health of pregnant women.

7. Conclusions

Getting the message across is a complex task, and largely depends on the desired goal. The medical and health community and environmental NGOs can play a particularly important role in engaging and influencing the public, as both these groups are well trusted and respected by the general public.

Regarding the position of the medical and health community as communicators for environment and health across society, the opportunity exists to leverage the esteem, trust and respect possessed by these professionals for a rational program in support of the prevention of harm and minimization of risk associated with toxic chemical substances. The challenge is to unify and manage the voice of these professionals in a manner that is both understood by the lay-person, and is also effective in conveying the environment and health imperative to policy-makers and regulators.

Environmental NGOs have a proven track record for influencing policy-makers and the public, and have many years' experience in working to reduce the environment and health risks associated with toxic chemicals.

In conclusion, coordination between the various groups active in the area is needed in order that best use is made of the available resources, and to ensure that communication strategies are based on the best information, are well framed, and properly targeted.

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ENVIRONMENTAL ETHICS AND FERTILITY IN EARLY CULTURES AND THE INDUSTRIAL AGE

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Summary

Archaeological excavations performed in various places around the world have shown that the earliest depictions portraying humans are feminine figures with exaggerated features of their sex. The feminine image has remained dominant in human culture, presenting deep continuities across space and time regarding her role as the mighty Mother of Nature, as the source of life. Striking commonalities recur in the way she was created and worshipped, in her symbols and ritual objects in diverse cultures from Stone and Bronze Ages to even more recent indigenous societies in Africa, parts of Asia, the Americas and Polynesia. Woman's profound experience of herself as a creator of life helped to define the image and the qualities of a Great Mother Goddess or Earth Mother Goddess, across time, as Nature herself, who could protect, nourish, contain and transform life in all its forms. This primordial concept of the Great Mother Goddess was the foundation of later prehistoric and proto-historic cultures in all parts of the world. Her cult was centered on fertility beliefs and rituals, executed on a seasonal basis to reinforce Nature's potential to increase reproduction of humans, animals and crops. Her identification with Nature led to the deification of natural and cosmic elements worshipped and respected as parts of her. Fertility was regarded as a holistic system in accordance with cosmic and natural elements ruled and controlled by her,

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 311–334.

strongly connected with environmental observations affecting reproduction's dynamic. The entire process of reproduction was ritualized, and its symbolism reveals how human consciousness primarily perceived the origins of life; also the manner and reason by which the source of life, when personified, was related either to images declaring environmental associations, or as such, proved to be essential in human wellbeing.

On the contrary today, due to man's dramatic change of attitude towards Nature as being devoid of sacredness, the environmental impact on fertility is considered within the general context of environmental ethics in the industrial age, whereby notions are interpreted as ethical, good or valuable according to how man places himself in relation to other living beings, and to the environment.

The current state of man's impact on the environment is discussed, specifically the dangers stemming from chemical pollution, in particular from endocrine disrupters and their deleterious effect on male and female fertility, leading to ethical problems. The role of governments, of non-governmental organizations and the public, which should play a more active role in the enforcement of precautionary measures, is stressed.

The recent ecological deterioration, affecting human health, cannot be dissociated from the steep economic gradient between industrialized and developing countries, which must be blunted as a prerequisite for world peace and prosperity.

1. Introduction

Early samples of artistic creation depict women to the greatest extent, and hardly any men. The creation of the female image has an enormous tradition in human culture. Primary depiction forms lasted for millennia; figurines of the Acheulian period, statuettes and bas-reliefs from the Upper Paleolithic and Neolithic Age emphasize generative features, pregnancy and some on giving birth (Preziosi, 1960). By the end of the last Ice Age (13.000-12.000 B.C.), a leading catalyst for the beginning of agriculture, human consciousness evolved dramatically, identifying gradually the feminine image as the umbilical cord connecting people with Nature. Later presentations from the Chalcolithic and Bronze Age give combined forms of the feminine archetype and her attributes, representing stars, plants and animals (Wolkstein and Kramer, 1983). Both periods indicate a background based on the observation of women's ability to create life that led gradually to religious formulations. Human consciousness, either as awareness or instinctive reflex, carries the whole experience of life on this planet since its formation. In the Stone and the

early Bronze Age, woman was believed to have a magical connection with the earth. Her fertility was identified with the fertility of life on earth. In the Neolithic Age she was thought to be the pattern to which the whole of life obeyed, from the circumpolar movement of the stars to those of the tiniest creatures.

Late Neolithic, Chalcolithic and Bronze Age female figures speak clearly of matrix-oriented religious system; written evidence confirms and specifies that the sacred feminine life creator was embodied by the image of a Mother Goddess, the fertile womb that gave birth to everything (Wolkstein and Kramer, 1983). She was revered as the Almighty Mother of the earth and cosmos; she was herself the earth and cosmos. She was the numinous Goddess within all her manifest forms.

Abounded shrines and temples were erected to her honor (Reade, 1991). Fertility rites were the center of her worship. Worship of fertility included fertility of all kinds: agricultural, animal and human fertility (Struhal, 1992). Most cultures performed periodical sexual fertility rites, believing that by having sexual intercourse practiced in the fields, crops could grow or flourish. Plants could reproduce their kind only through the stimulation of the union of male and female, by their real or mock marriage (Frazer, 1998). Key element in this practice was the exercise of "sympathetic magic", evoking magic in the form of imitation, meaning that when humans are sexually active then gods are sexually active too. Sexual activity of gods in turn ensured fertility of animals and crops.

The female principle of life was the earth or womb, out of which crops grew; thus the male element was thought to be supportive and secondary (Dalley, 1998). Public mating of a male member of the community with a female representative of the Earth Mother Goddess was a common ritual held mostly in the fields or in her temples (Frazer, 1998). Earth Mother Goddess or Mother Goddess religions preserved their character up to the Iron Age, which began about 1200 B.C. Deification of Nature and fertility was attributed now to the male element as well (Tubb, 1998). Both female and male personifications of Nature had complementary roles in the process of creation, but it was only the great goddesses of the Bronze Age that were still worshipped as late as Roman times, in the West, although in Asia, Far East, Central and South America, Africa and Polynesia, Earth Mother Goddess cults are still practiced, enhanced by various local beliefs.

In the long period preceding the Modern Age, man's relation to Nature changed fundamentally due to the introduction of the scientific approach that led to the development of rampant technology and the belief that man is in control of the world. Extreme exploitation of Nature to man's own benefit followed this

anthropocentric arrogance. Environmental ethics have then progressed admirably, often following a philosophically abstract path; however, they are increasingly confronted with pressing environmental attacks, threatening the well being, and potentially, the survival of man. Environmental ethics certainly affects our reactions to environmental threats. However, the variety of prevailing ethical viewpoints hinders the development of uniform policy and action to these threats.

If man is indeed the protagonist on this earth and if his well being, the preservation of the life-style of a bulk population depends on the exploitation of Nature's resources leading to deforestation, species extinction, etc., then such actions are "ethical", valuable, good and right.

If man is just one species – surely on top of the evolutionary ladder amongst the millions – and regards the variability in Nature as a gift and a precious component of his presence in this world, then deforestation, species extinction, etc., are bad, immoral, wrong. However, if deforestation could help in rising the economic status of a population to an affluence that could channel wealth to introduce environmental friendly technology, then a decision on what is ethical, good, valuable, could be difficult.

If the increase of the planet's inhabitants to levels compromising the world's food and leading to catastrophic air and water pollution were realistic, then family planning of various sorts, including abortions, could be valuable and perhaps justified. But if man is destined to" multiply and conquer the world" or if a family in a developing country needs hands to work in the farm or fish for food, then family planning is bad and wrong.

The aim of this chapter is to show how human consciousness initially perceived the environment as a sacred whole, considering man as a part of it. Focus was given to increasing reproduction whereas today, the human impact on the environment is harming it, mainly due to the danger of chemical pollution and its pestiferous effect on fertility. And given man's current detachment from Nature, any evaluation of his impact on the environment only leads to a complex maze of ethical dilemmas.

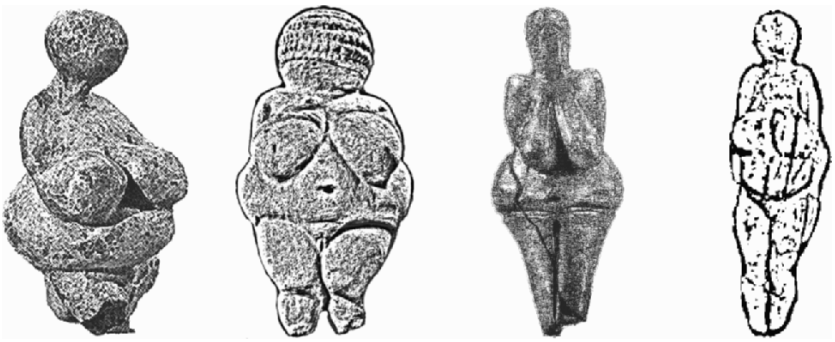
2. Acheulian period

Regardless of place, stage, and variety of tools, it is first the feminine figure that comes from the very remote past, carrying the visions of our distant ancestors. Her history is traced in the very early specimen of artistic creation, and reveals her route over thousands of years in human culture. She appears with the beginning of art, in

the first efforts to carve stone in Africa and Middle East. The oldest known figurine in the world is 330.000 years old, attributed either to Homo erectus or Archaic Homo sapiens. It was found between two layers of volcanic flow, aging 800.000 to 233.000, at Berekhat Ram on Golan Heights (present Israel) (Marshack, 1995). It was carved out of a piece of basaltic tuff and represents a 35 mm great–breasted female figure with featureless head (Marshack, 1997). She is considered to be a fertility symbol, named the Acheulian Berekhat Ram figurine or Goddess. Another figurine 58.2 mm long, with a slight female form and modified by precisely the same treatment is the Tan-Tan Venus found in southern Morocco. It was made of a moderately metamorphosed quartzite and discovered in sediments of Middle Acheulian deposit, aging 500.000-300.000 years (Bednarick, 2003).

3. Palaeolithic Age

Her traces are lost for an enormous period of time, till she is found again in the Upper Paleolithic. She appears in a most prominent way, created as a divine robust figure with emphasis on its genetic characteristics, with or without synchronous symbolic codices, defining her manifestations. A reasonable number of more than 200 female figurines with exaggerated corpulence are found over a broad geographical area ranging from France to Siberia.



a. Venus of Gagarino b. Venus of Willendorf c. Venus of Dolni Vestonice d. Venus of Avdeevo

Figure 17. Paleolithic figurines a. Venus of Gagarino, b. Venus of Willendorf, c.Venus of Dolni Vestonice and d. Venus of Avdeevo (drawn by Alexandra Katsivelaki from the original Museum statuettes).

They were carved from stone, bone and mammoth ivory and are depicted having enormous breasts, protruding belly, very pronounced vulva, prolonged buttocks and thighs, tapering legs and no facial features (Sandars, 1992).

They date from about 30,000 B.C. to 11.000 B.C. And are known as the "Venuses" of the places found (Pfeiffer, 1982); of Dolni Vestonice (Figure 17c), Willendorf (Figure 17b), Laussel, Lespugue, Monpazier, Sireil, Savignano, Balzi Rossi, Gagarino (Figure 17a), Kostienki, Avdeevo (Figure 17d) (Gvozdover, 1995), and others. Most of them depict mature women in various stages of the reproductive cycle: either not pregnant or in various stages of pregnancy. The basic forms are common; they are very small items, focusing mainly on generative organs, no matter the distance of their origin. They are considered to be cultic objects, idols or amulets reflecting ideas of maternity and fertility, implying a religious belief strongly connected with fertility.

Venus of Laussel, a Perigordian limestone bas-relief, was carved at the entrance of an overhang cave in Dordogne, South France (Lalanne, 1912). She is graven on a fries, together with other women and only one man, keeping one hand on her protruded belly, possible pregnancy, while the other holds a bull or bison horn in the form of a crescent moon (Giedion, 1962). Her fingers point towards her vulva, her head inclines towards the horn or crescent that has 13 vertical cuts representing possibly the 13 lunar months in a solar year, or the thirteen days of the first crescent to the full moon (Marshack, 1972). She retains traces of red ocher indicating most probably the correspondence between the lunar phases and menstruation (Knight, 1991). Dating between 27.000- 22.000 B.C. she was created by hunter-gatherers closely attuned and dependant on the cycles of seasons, and therefore able to observe that monthly bleeding was connected to special lunar phases. The cave was potentially a ritual place where women could gather in safety away from their group, presuming that in close proximity their menstrual cycles would possibly synchronize and thus facilitate their nomadic wandering.

4. Neolithic Age

In the Neolithic, a deep relation was formed among people with the earth through sowing, tending and harvesting the crops and breeding domestic animals. Plow and wheel were invented then, around 5.500 B.C. in fertile valleys of Mesopotamia (Balter, 2005). Woman was believed to have a strong connection with the earth, cosmos, and the rhythm of Nature. A Mother Goddess-oriented cosmology was

invented by all cultures of that time. Many images, felt to belong or to describe her, were made at that time.

Excavations in Europe, the Near East and at Asikli, Çatal Hüyük and Hacilar in Anatolia, Mesopotamia, the Indus Valley, China and South America brought cultures to light, dating as early as 8.000-2.700 B.C. (the Neolithic was not experienced at the same time throughout the world), echoing a strong relationship with an almighty Mother Goddess. She was a Mother Goddess with many aspects, concentrating in her the profoundly experienced process of birth, death and regeneration**.** Sky, earth, and underworld were unified in her being (Kramer, 1963). Animals, serpents and birds were all her epiphanies, expressions of her hypostasis (Campbell, 1983). Plants and trees were escorting her image, attributes of her nature. Mountains, hills and groves became sacred, and were devoted to her; springs and wells became places of her blessed healing. This was the phase in human evolution when magical rituals were established to please her wishes and demands, to reinforce her fertility and to propitiate the Goddess with offerings that would bring protection and abundance.

Countless feminine images and ritual objects from the Neolithic, relics of her cult keep coming to light all over the world showing a steadily amazing similarity in form and concept (Ucko, 1968).

A terracotta statuette of the Mother Goddess, dating between 6800-5000 B.C (Mellaart, 1965), was found in Çatal Hüyük, a large Neolithic settlement, near Konya in Turkey. It is considered, after Jericho, to be the world's oldest village 9000 years old, with (approx.) 10,000 inhabitants. It was a shocking discovery, since it begged the questions of how, or even why, this period's rudimentary agriculture led so many people to gather in houses, abandoning their hunting and gathering nomadic life in the processes. Clay figurines of obese women were excavated in a temple at the site (Mellaart, 1975). It is the earliest figure found to portray a female sitting on a throne, guarded protectively on both sides by two leopards. She was found in a shrine, located in a grain bin, and is depicted in the process of giving birth; the head of the baby is visible, coming out of her tummy (Balter, 2005). Bullhorns were placed all around her. Bullhorns were found in all houses of Çatal Hüyük, laid in a ritual way (Mellaart, 1965). It is interesting that bullhorns were always connected with the Goddesses of fertility of that time, as well as later periods, and over extended geographic areas of Europe, Africa and Asia.

In the period 6500-5500 B.C. a farming society appeared in Northern Mesopotamia and Syria**,** known as the Halaf Culture, that gave numerous terracotta or unbaked

clay female figurines made for religious or magical purposes (Collon, 1995) connected with fertility. They display strong stylization with emphasis on sexual features. Their faces are pinched out to form a large nose or chin. They sit with exaggerated thighs extended, supporting heavy breasts with their arms (Reade, 1991). Their stylized depiction of the feminine form, standing or seated, remained an artistic convention for several millennia in Northern Mesopotamia, Syria, Anatolia, Egypt and the Aegean.

The late Neolithic period displayed more objects of the divine feminine image. The Greek islands offered marble to the stone-age carvers who created marble figures between 4500-400 B.C., the Cycladic idols (Fitton, 1999) with abundant flesh, swelling lines suggesting fertility, and nourishment. Their hands are joined under their breasts, keeping to the old tradition of the area.

The Ubaid culture from Ur in Southern Iraq produced the so-called "Lizard" figurines of about 4500 B.C., portraying women often suckling a child (Wooley, 1995). Their name is owed to their stylized type lizard head (Collon, 1995). Found in the cemeteries of Ur and Eridu, they represent symbols of fertility or votive objects (Reade, 1991).

Amuq Plain, a fertile area in Anatolia that attracted farmers for thousands of years attributed figurines from around 4500 B.C., which once again emphasize sexual features, large breasts and heavy thighs (Collon, 1995).

At 4000 B.C. the Badarian culture, in predynastic Egypt, gave female images found in burials, focusing on eyes, breast, pubic area and hips (Spencer, 1993). Their hands were kept on their bellies, made out of stone, hippopotamus bone, wood and clay. Their association with fertility was linked with the rebirth and regeneration of the deceased in the Afterlife. Naqada's burial mound discoveries revealed naked female representations with emphasized sexual organs and large striking eyes. (Shaw and Nicholson, 1995) They are thought to have provided magical support for rebirth and regeneration as well (Hart, 1991).

Around 4000-3500 B.C. ceramic figurines appeared along the coast of Ecuador at Valdivian sites, the earliest in the Americas. They continued the region's earlier stone figurine tradition. The majority is female, but there are some displaying both female and male attributes. Emphasis falls again on breasts and sexual organs that were only the ones depicted bare. They represented fertility figures, serving reproductive purposes (Metropolitan Museum). They were used in unknown fertility rituals carried out at harvest time.

The same form of depiction is also to be seen in a China Venus, a 6.7 cm jade figurine from the Hongshan Neolithic culture dating from 4500 to 2250 B.C. The Hongshan were located between Inner Mongolia and present day's Liaoning and Hebei provinces (Nelson, 1995). The amazing sophisticated jade carving presents a female again, with enormous breasts, supported by her hands and pronounced belly.

Before the rise of Indus civilization, female terracotta figurines in various shapes and forms were made at Mehrgarh (7000-2000 B.C.), at periods between 7000-4800 B.C. on the hills of Baluchistan in India. They were the first in South Asia, and portray seated nude deities with exaggerated breasts and buttocks and exquisite hairdressing (Jarrige, 1988). They were used in fertility rituals to ensure fecundity of humans, animals and a rich harvest.

The Altyn-depe settlement in today's Turkmenistan offers, in the late third millennium, high-quality clay terracotta figurines, excavated close to or inside tombs, related to ideas of fertility and rebirth (Masson, 1998). They carry high headdresses; they have realistically formed breasts. Triangles indicate outstretched arms. Their long, narrow waists lead to broad hips. Their lower halves are bent, creating the impression of a seated pose.

Chalcolithic Cyprus at the end of the third millennium (Tatton-Brown, 1997) gives a remarkable terracotta image related to local fertility cults, representing a goddess or a woman milking both her breasts into a basin placed on her thighs (Louvre Museum).

The late Chalcolithic and Bronze Ages provide us with additional information about the character of the Mother Goddess, since written sources from these times have been decoded and read.

5. Bronze Age

It is only from the Bronze Age that hymns were discovered addressing the great Goddesses of Sumer and Egypt, inscribed on the sun-baked clay tablets of the Sumer or in hieroglyphs on the walls of Egyptian temples (Wilkinson, 2000). They reveal a rich mythology concerning the images of the Mother Goddess that carries forth Neolithic traditions enhanced by contemporary attitudes towards Nature and social values (Black and Green, 1992). It is in the Bronze Age that the feeling for the sacredness of Nature is clearly expressed in words and transmitted through prayers to the goddesses or through the voices of the goddesses themselves. They announce themselves to be the fertile womb, which eternally regenerates plants,

animals, human beings; the life force, which attracts the male to the female; the power that creates, destroys and transforms itself (Harper, 1901).

Various goddesses, variants of the Great Mother Goddess, are presented as the source and embodiment of all instinctive processes: Inanna, Ishtar, Astarte, Anat, Cybele and Hathor are the supreme Goddesses of Sumerian, Babylonians, Phoenicians, Canaanites and Egyptians (Sags, 1995a)**.** They are few among many other goddesses of love, sexuality, motherhood and fertility of men, animal and crops all over the world (Kramer, 1963). They were complex goddesses, and many of them also had martial aspects. They were the benevolent Almighty Mother Goddesses but also tremendous warriors against their enemies.

In India, before the Aryan invasions around the 14th century B.C, the Vedic sages described with amazing clarity their image of the Mother Goddess in the magnificent poetry of the Vedas and the Upanishads, expressing a deeply passionate devotion to her (Brians *et al.,* 1999). In the northern areas, people named their great mountains in her honor and worshipped her as the dynamism of the creative principle, always seen in an eternal embrace with her divine consort.

5.1. Inanna, a Sumerian goddess of fertility

Inanna was the most important goddess of the Sumerian pantheon, a goddess of love, fertility and war. Variations of her name mean either "queen of the sky" or personification of the planet Venus. Her symbol was the eight-pointed star, representing the planet Venus (Dalley, 1998). Her Temples were in Uruk, Zabalam and Babylon. She was a goddess associated, in terms of symbolism, with the crescent moon, the planet Venus and the serpent. Wings and serpents adorned her shoulders, traces of the ancient Neolithic Bird and Snake Goddesses**.** The goddess figures prominently in various myths, such as in the Cycle of Innana, a collection of poems concerning her in her life and death relation to her brother and lover, the vegetation-god Dumuzi or Tammuz*;* also figuring her in the magnificent lunar myth of her descent and return from the underworld that was inscribed on clay tablets at around 1750 B.C. (Wolkstein and Kramer, 1983).

In this particular myth she travels to the realm of the dead and claims to be its ruler. Her entry to the underworld was celebrated by the onset of the after harvest period. Her sister Ereshkigal, who rules the place, sentences her to death. With Inanna's death, however, Nature died with her and nothing would grow anymore. Through the intervention of the god Enki she could only be reborn if another person would take her place. Inanna chooses her beloved consort Dumuzi. She attends the death

rites of the Sacred Bull of Heaven, and then she comes back to the earth. After her return, Dumuzi is chased by demons and loses his possessions, his genitals and his life (Wolkstein and Kramer, 1983). Male principle was viewed as periodically being slaughtered and buried, planted as seed to ensure the success of seasonal planting. Inanna laments her decision and searches for him. She finds him and ensures his resurrection, so that he could be back for half a year to enable fertility of womb and soil. This myth has some relations to the Greek Demeter myth as well as to Celtic beliefs.

During the time of growth, that was autumn in the Near East when the first rain after the long summer fell, the Sumer celebrated the "Hieros Gamos" (Sacred Marriage) of Inanna and Dumuzi singing ecstatic, erotic hymns to encourage their union. It took place yearly, at the autumnal equinox, as the New Year's Festival that brought the land fertility and growth again. This was due to Dumuzi's returning from the underworld and making love with Inanna again (Kramer, 1969).

Being explicitly a goddess of sexuality and fertility, Inanna's worship included sacred rites to potentiate the sexual activity of the goddess. Early fertility cults had incorporated in their ceremonies a variety of ritualized sexual behaviors involving priests, priestesses and worshipers (Frazer, 1998).

The first written law system, the Code of Hammurabi created between 1792 - 1750 B.C. and now in Louvre Museum, certifies the roles of the priestesses. Law 181 refers to the dowry rights of four types of female cultic personnel: the hierodules, sacred prostitutes, lay priestesses and devotees to goddess's temples. In laws 144 - 147 all types of sacred women appear to be highly protected.

During and after the decline of the Sumerian kingdom, she was replaced by the Semitic goddess Ishtar who became an incarnation of Inanna to be invoked at Inanna's original temples at the cities of Erech, Kish and Ur.

5.2. Ishtar, a Mesopotamian goddess of fertility

Ishtar was the supreme Mesopotamian Goddess, the Akkadian/ Babylonian Great Goddess of sexuality, fertility and warfare. She is a later and more complex development of Inanna whose myths were adapted in her worship. Apart from their common aspects she became the one bestowing the ancient kings with the right to rule over her people. She is depicted with symbols of fertility such as the date palm, with the planet Venus on her crown as an eight-pointed star, and the crescent moon (Collon, 1987). She appears often on relief carvings and seals as a strong warrior, or

as an almighty Mother Goddess. The Hittite and Hurrian people of Anatolia, Sumer, Egyptians and the Assyrians revered her in grandiose temples at Nineveh, Arbela, and Uruk (Layard, 1849). The most important street in Babylon was the Processional Way leading from the inner city through the Ishtar Gate to Bit Akitu "the House of the New Year's Festival". The world famous Ishtar Gate was a glazed-brick building decorated with images of bulls and dragons. North of the gate, the street was lined with figures of striding lions, sacred animal of Ishtar, to serve as guides for the ritual processions to the Temple (Marzahn, 1992). She was the Scharrat Schame (Queen of Heaven) (Wolkstein and Kramer, 1983) and the mother who gave birth to the world "She Who Begets All". She was renowned for her power of creation, desire, prophesy, healing and divine rulership (Luckenbill, 1927). She is described in texts as a "beautiful figure" having "sweet lips" and Babylonian scriptures call her "Torch of Heaven and Earth", "Opener of the Womb", "Exalted Light of Heaven".

5.3. Hathor and Isis, Egyptian goddesses of fertility

The main pro-dynastic Egyptian goddess associated with love, birth and rebirth was Hathor. She appeared as a woman with a sun disk between a pair of cow horns on her head, with or without a pair of feathers (Quirke, 1992); another incarnation was that of a woman with a cow's head, or as a cow with the solar disk. She was worshipped in her role as goddess of fertility, women and childbirth at Dendera. At Thebes she was regarded as goddess of the dead, as "Lady of the West", associated with the sun god Re on his descent below the western horizon. Her cult eventually merged into the one of Isis, the prominent fertility deity and divine mother of later periods. Isis revived her consort, the slaughtered Osiris for long enough to conceive a child (Putnam, 1990). She was often depicted with her child Horus on her lap and by extension regarded as mother and protectress of the pharaohs'.

Renewal of fertility and regeneration of life for both living and dead were important concepts in Egyptian religion. Kingship and priests performed daily rituals to maintain the balance of the Universe, while ordinary people worshipped their gods at home shrines and religious festivals (Shafer, 1991). Isis' larger temple is at Philae in the Nile. Her worship spread outside Egypt to Asia Minor, Greece, and Italy. Temples of Isis were built in Galatia, Spain and along the rivers Danube and Rhine.

6. The goddess Kuan Yin

In Chinese mythology there was a Mother Goddess who existed before heaven and earth. Her image was connected with the oral inheritance to the Bronze Age and the shaman tradition, which later evolved into Taoism. Legends describe her as the Mother, or Grandmother; the cosmic womb of all life, the gateway of heaven and earth (Kohn, 1993).

Taoism kept alive the feeling of relationship with Nature, of being a primordial mother**.** The essence of Taoism is expressed in the Tao Te Ching (Kohn, 1993). The word Tao means the fathomless Source, the One, the Deep. Te is the way the Tao comes into being, growing like a plant from the ground. Ching is the slow shaping of that growth through the activity of a creative intelligence, expressed as the organic patterning of all life. The Mother Goddess was particularly close to women, who prayed to her for a safe child delivery, the protection of their families and the healing of sickness. She was present in their homes, shrines and temples, on the sacred mountains, in the valleys and vast forests. Yet, like the goddesses in other early cultures, she also had cosmic dimensions. She was the Spirit of Life itself, the Protectress of Life, and above all she was the embodiment of love, compassion and wisdom (Brians *et al.,* 1999). Although she had many names and images these eventually merged into one goddess who was called Kuan Yin (she who hears, she who listens), whose cult spread all over China, Korea and Japan and exists still today.

7. Nature elements as symbols of the Mother Goddesses

A group of images was associated with the Great Earth Mother Goddess as the creator and transformer of life. The cave was her womb, the place where the tribes held their sacred rites in the Stone Age (Marshack, 1972). This was extended to gardens, forest glades and certainly temple precincts, as civilizations developed later. The circle, triangle and the egg-like oval described her womb and her vulva. Other forms like the wavy line, the meander and the spiral were, as early as the Paleolithic, connected with her. These were found on the walls of the caves, on rocks, stones and dolmens (Pfeiffer, 1982). The spiral form is intrinsic to water. The wavy lines were the rain and river water. The rivers, that have their origin in the mountains and end in the sea, moving at their own pace following a meandering course, resemble the coils of an enormous serpent moving across the earth. The serpent-like spiral, the meander and the labyrinth were the hidden pathways of the life force. Later from the Neolithic, rounded or egg-shaped pottery vessels symbolized her body. These basic forms, so familiar to ancient cultures everywhere,

trace their descent through subsequent civilizations all over the world, East and West. It is from the Neolithic era that we have inherited all the images related to her, presenting her as the one who obtains the energy to bring life into being, maintain and transform it, and potentiate rebirth or regeneration. The moon is perhaps her most ancient symbol but the sun and the planet Venus were always escorting her image in later depictions during the Metal Age. The association between the changing phases of the moon, the seasons of the year and the woman's life cycle was the foundation of a mythology inspired by the moon. Organic life on this planet is strongly influenced by the magnetism of the moon and in the Bronze Age mythology of Sumer and India, the Mother Goddess was imagined as the primordial watery abyss, personified by a great serpent. The association of water and goddess is very strong and she is imagined as the Water of Life, or the one who offers it (Wolkstein and Kramer, 1983). Fertility Goddess Kuan Yin in the Far East is also goddess of the sea, and protects all who sail on her. Greek Aphrodite was born from the sea foam. Isis was called Star of the Sea. In both East and West shrines to the goddess were built at the springs, and at certain times of the year were ritually decorated to invoke the continued blessing of the Mother Goddess.

Three animals in particular always signified her presence or power: the cow, lion and the snake (Campbell, 1983). Anthropomorphic and animal features often melted in fertility goddess (James and Davies, 1983). Inanna in Sumer, Hathor and Isis in Egypt were called "the Great Cow" or "Celestial Cow". Her temples in Sumer were adorned with enormous horns. Hathor was often depicted as a woman with a cow's head (Pinch, 1993). Both Egyptian goddesses were depicted crowned with bullhorns surrounding the disk of the sun or the moon (Reade, 1991). Images of Ishtar in Babylon, Durga in India and Anat in Egypt stand on a lion. Cybele rode on a chariot drawn by lions. The Earth Mother Goddess of Minoan Crete carries snakes on her bare torso and hands, and is praised as "Lady of the Beasts". The snake had a very important role, but also other associations. The snake and its abstract derivative, the spiral dominated religious art during the Neolithic and Chalcolithic periods (Gimbutas, 1974). It is an age-old symbol of healing. Important were also many birds sacred to the goddess in the Neolithic, up to Roman times; amongst them, the crane, the swan, the goose, duck, owl, as well as smaller birds, like the dove and the swallow. The bee and the butterfly also belonged to the mythology of the Mother Goddess (Sakelarakis, 1989). There were also some other animals that became associated with her variants, because of their abundant fertility: rabbit for Aphrodite and the Mayan Ixchel, and the sow for Demeter.

There are many images of food and nourishment that have always belonged to the mythology of the Earth Mother Goddess. The Tree of Life stands prominently in this

chain of images (Woolley and Moorey, 1982). A tree in many different cultures was sacred to the goddess; sacred trees were planted in the precincts or inside her temples in Mesopotamia, Egypt, Crete, India and China. Wheat, barley, corn and pomegranates were part of her rituals, or connected with her various depictions (Saggs, 1995).

The Great Earth Mother Goddess, whether as Nature without or within, had a beneficent, nurturing, supportive aspect.

Demeter in Greece, and Ceres in Rome, are the last goddesses in the West to remind us aspects of her: mostly the ancient connection between the Mother Goddess, the Earth and the food the earth offers. Greek and Roman goddesses, although they had moved closer to the concerns of civilization, still carried through the cosmic dimensions of the older Mother Goddesses.

8. The transition to the Industrial Age

The transition from the deification of natural and cosmic elements to their examination through the philosophical approach is a supreme intellectual accomplishment of the ancient world. Presocratic philosophy, for the first time, postulated natural elements as the cosmic material comprising the universe, whereas Hippocrates formulated and systemized the environmental impact on health.

The philosophical approach, however, was reduced to silence in the Middle Ages by theocratic doctrines and dogmatism.

The Enlightenment revived the quest for knowledge, and the introduction, as well as the application, of the experimental method as a means to understand and manipulate physical phenomena, led to explosive advances in Technology, heralding however the deleterious effects of man on his environment.

9. Industrial Age

Man's predicament is his own nature, his unlimited trend to explore the unknown, to unlock the earth's mysteries and to apply without restraint the acquired knowledge (Razis, 1996). The horrendous effects of our society's activities on the environment, the continuous reduction of unspoiled land in favor of cities and infrastructures, the unendless increase in transport modalities and in industrial output, including those of life-saving pharmaceutical establishments, result from man's ingenuity and

creativity. However, this unlimited growth can be man's predicament. Focusing on the chemical industry, the synthesis of thousands of chemicals for a variety of applications satisfies man's thirst for more leisure at home, for ever more rapid transportation by speedier cars and planes, for better communication, for more food, less pain, less disease. However, this huge increase in new molecules coming directly or indirectly in contact with humans, animals and plants, is a constant threat to man and his environment.

Among the chemicals that pose a major threat on health and the environment are the endocrine disrupters (Nicolopoulou-Stamati *et al.,* 2001). These agents, affecting mainly reproduction, derange the endocrine system in various ways, by competing with endogenous hormones for binding sites on the respective receptor proteins, and by affecting the synthesis, secretion, transport or catabolism of the hormones. The endocrine disrupters mainly affect steroid hormones, and as they represent principal regulators of growth, developmental and metabolic processes, the consequences of their disruption are grave, especially on fertility (McLachlan *et al.,* 2001; Brevini *et al.,* 2005). Several chemically unrelated substances belong to the category of endocrine disrupters (Soto *et al.,* 1995). Human exposure can occur in a variety of ways, by food or water intake, by skin absorption or inhalation. However, for the majority of chemicals, the main source of exposure is by food. Particularly endangered are the foetuses by way of the placenta, as has been demonstrated by blood samples from the umbilical cord, identifying therein DDT, hexachlorbenzene, PCBs, DDE and dioxins, amongst many other toxic substances. These chemicals have also been detected in the mother's milk and can be passed to the nursing infants (placenta, milk).

The developing organism has not developed through the ages the effective mechanisms that would provide protection against toxic chemicals and, indeed, growth retardation, behavioral problems such as poor attention, hyperactivity and decrease in the intelligence quotient have been observed in children. This was due to exposure to dioxins, PCBs and other endocrine disrupters, but also to toxic substances, such as lead and methyl mercury, either in the womb, or during early childhood. Effects on the immune system have also been reported after exposure to DDT and DES, and the role of endocrine disrupters in carcinogenesis has received due attention (Bagga *et al.,* 2000; Romieu *et al.,* 2000; Kogevinas *et al.,* 2001).

Female and male reproductive processes, intricately controlled by the interplay of several steroid and protein hormones, are strongly affected by endocrine disrupters. The effects on fertility of endocrine disruption were recognized initially by chance observations (Colborn *et al.,* 1993; Oetken *et al.,* 2004); later by animal

experimentation and epidemiological studies. After administration of high doses of estrogenic chemicals to rats, reduced fertility and structural abnormalities of the reproductive system was observed; the same observations were made after exposure of the animals to very low levels of dioxins in the wombs.

The use of the synthetic estrogen DES in the 1950's as a drug combating spontaneous abortions of pregnant women led to the birth of girls which later developed vaginal cancer, reduced fertility and structural abnormalities of their reproductive system (Herbst *et al.,* 1971; Swan, 2000). Genital defects were also observed in the male offspring (Gillet *et al.,* 1977; Stillman, 1982).

A considerable increase of several reproductive disorders has been described in males during the last years. Significant is the decrease of sperm count and motility (Carlsen *et al.,* 1992; Auger *et al.,* 1995; Adamopoulos *et al.,* 1996; Storgaard *et al.,* 2002), the increase of boys born with genital abnormalities, e.g. cryptorchidism (Skakkebaek *et al.,* 2001) and the increase in testicular and prostate cancer. It is believed that these disorders have their beginnings during the fetal and early life due to the presence of endocrine disrupters in the environment. Recently, it was demonstrated that two widely used toxic compounds, vinclozolin and methoxychlor, a fungicide and a pesticide, respectively, show endocrine disrupter effects (Anway *et al.,* 2005), the first by blocking the action of androgens at the level of the androgen receptor, the second acting as an estrogen. They cause fertility defects in male rats, which are passed down to nearly every male in the subsequent generation. This is reminiscent of the action of DES on the offspring of pregnant, previously discussed.

The alarming decrease in fertility, particularly in many Western developed countries, has rendered *in vitro* fertilization a routine procedure, something very new for mankind.

Although this mode of procreation is now accepted the world over, it raises major ethical problems. For that part of humanity which regards the fertilized ovum as a human being endowed with a soul, the destruction of unused super-numerous embryos is equal to manslaughter. Although the reduction of ova used in each attempt of IVF was introduced to overcome this problem, it has not basically eliminated it. Furthermore, the psychosomatic union of the parents attained in the act of procreation, which is regarded by a part of the population as extremely important for the seminal event of bringing children to life, is absent in IVF. Although it is generally assumed that children born by IVF are in no way medically inferior compared to those born by the natural way, some reports have indeed

suggested an increase in the occurrence of certain genetic diseases in the former category.

The sensational biological breakthrough of the cloning of an animal by the transfer of a somatic nucleus to an enucleated ovum, negating the dogma of the irreversibility of the differentiated state, has led to the application of this procedure for the cloning of a variety of mammals. The possibility of cloning human beings, although universally rejected and condemned as unethical, nevertheless is a potential possibility that could cross the ethical barriers, provided the overcoming of the medical problems observed in many of the cloned animals. It is worth remembering the initial outcries against IVF, which abated after the ascertainment that the method is medically safe, without causing late effects in treated women.

10. Approach to the problem

Two main approaches for the protection of people's health from the hazardous substances synthesized en masse by the chemical industry are now followed: prevention and regulation. Prevention from harmful effects of chemicals involves measures to ban the production and use of such compounds. It implies, however, that these have already exerted their harmful effects, and thus have been recognized as dangerous to public health. On the other hand, regulation of the production and release of chemicals necessitates the knowledge of the potential effects of the agents on human health, and the allowable rates of release of a chemical, i.e. risk assessment (Hens, 2001). This methodology is riddled with uncertainties and limitations, but is nevertheless serving to significantly reduce the release of harmful chemicals in the environment. To overcome the problems inherent in this approach, the Precautionary Principle has been introduced, requiring that chemicals are to be released in the environment only if they have been proven harmless. Although this represents an ideal principle, its application to the thousands of chemicals, many with potential endocrine disrupter action, is a time consuming and almost Herculean task. Furthermore, in the field of pharmaceutics, it can lead to the delay in the use of potentially beneficial drugs. In spite of the difficulties in its application, the Precautionary Principle should be a goal to be strived for.

It is quite unfortunate that the people recognizing the dangers stemming from chemical pollution and other environmental problems have little power to enforce the obvious remedies. This in spite of the various United Nations' resolutions proclaiming the fundamental right of man to adequate conditions of life in an environment of a quality that permits a life of dignity and well being, along with

many other resolutions of various agencies to preserve the ecosystem and ensure a healthy environment. A classical case illustrating this impasse is the refusal of the United States – the sole superpower with unique possibilities to shape world events - to undersign the Kyoto agreement, which, if enforced, could provide a hope for avoiding the catastrophes of global warming. Nevertheless, steps to combat environmental hazards facing, not only industrialized, but also developing nations, could be implemented by regional authorities, better amenable to a knowledgeable public influence.

Europe could be an example, and hope for implementing measures to tackle the increasing health and environmental dangers of chemical pollution. The European Union's policy on health and environment is straightforward, stresses the need to preserve it and to promote measures to deal with regional but also worldwide environmental problems, and has introduced legislation in this direction. Although the European industrial lobby is not insensitive, it is a hindering factor on the implementation of the proposed measures by influencing governmental decisions. Important is the mobilization of public awareness by scientists and group of informed laymen, known for their combativeness and drive, with the goal to apply pressure on their national decision markers, but also intergovernmental and international organizations controlling global health and environmental policy convergence, for more effective and drastic legislation.

Although the national government is the "protector" of its state's environment, it is under the highest pressure for legitimization by the general public and the media, and in this respect the role of non-governmental organizations, along with other non-governmental agencies, is invaluable.

11. Conclusions

During the first periods of human evolution, broadly defined as the Paleolithic and Neolithic eras, humanity lived instinctively as the child of a Mother Goddess, the personification of deified Nature and Cosmos, and knew life and death as two modes of her divine reality. Through the ages humans developed a different relation to their environment based on their critic approach to physical phenomena and on the experience of their innovative potential.

Man is the sole species capable of shaping his destiny. It is therefore crucial to understand why he is following now a path jeopardizing his existence and endangering his survival. One seminal answer should be sought in his human nature. History, past and present, teaches us that man's path on earth was a series of great

achievements, but also of horrendous acts performed in periods of brutal, totalitarian regimes, but also in periods of democracy, such as the present one. Dominance and survival were, and are, the main forces in these acts. The minority of this world, bases its well being on dominance, whereas the majority is still in the phase of survival. Both antagonistic worlds, contribute, for varying reasons, to the problem of environmental deterioration, increasing pollution, compromising health, the ecosystem, biodiversity, and the values of life on this planet. The new millennium is witnessing the advent of globalization, a system not so new or recent, which is generating unprecedented wealth; however, a mal-distributed one. In part as a consequence of this system, one and a half billion people still live in conditions of extreme poverty. Environmental dumping, recent technological and social developments in agriculture, the creation of a global consumer culture, are major mechanisms linking issues of global growth to poverty and ecology.

The reversal of the current ecological deterioration passes through the blunting of the steep economic gradient between industrialized and developing countries, a prerequisite for world peace, in which a humane regime of global regulations is a prerequisite, and is urgently needed (Razis, 2003). If the values of life on this planet, the ecosystem, diversity, environmental ethics, cannot be accepted, treasured and incorporated in our socioeconomic systems, if the world frantically continues an ever-growing pace of unsustainable growth taxing our health and the environment, if it fails to understand that a viable equilibrium must be established between growth and quality of life (human and of the environment), then the future looks bleak. If societies cannot prevent forthcoming events threatening man himself, they will be forced to curatively cope with the ensuing calamities. The challenge, in the historical perspective, has a don quixotic taint: education, public awareness, changes of attitudes, life styles and involvement of youth are important ingredients in the fight for an environment supporting peace and progress for all human beings (Mouzelis, 2003).

As reproductive health is essential for the maintenance of the human being, it is obvious that we have to reconsider our attitudes, and behaviour, to environmental issues. Current ethics should be revaluated to assure human survival and evolution in an environment not threatened by man's own arrogance and aggressive actions.

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SECTION 5:

REGIONAL ASPECTS

REPRODUCTIVE HEALTH PROBLEMS IN THE GREATER ATHENS AREA

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Summary

A number of reproductive health parameters have been evaluated in people living in the Greater Athens area. These include:

- changes in important seminal characters of men investigated for subfertility,
- the ratio of male to female newborn infants delivered in a busy maternity hospital over a period of 35 years,
- the incidence of premature menopause in a large cohort of Athenian women investigated recently,
- public awareness of gonadal decline in ageing men and its importance in their well-being.

The findings are:

1. A declining trend for seminal volume ($p<0.01$) and sperm number ($P<0.001$) was observed in a representative sample of 2385 Athenian men investigated during the period 1977 to 1993.
2. A decreasing trend in the ratio of male to female infants ($P<0.01$) was documented in 221.799 births recorded during the period 1969-2003 in this hospital.

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 337–352.

3. A higher incidence of perinatal care needs and raised in male newborns and more premature mortality in male neonates than in girls.

4. A high percentage of Athenian women (22.2%) who fail to reach the age of natural menopause, with 10.5% experiencing menopause before the age of 40 years.

5. A low public awareness of reproductive health problems in Athenian men over 50 years of age. Those, however, who were properly informed showed a better physical, mental and sexual activity.

Overall the data show that certain aspects of reproductive health have markedly deteriorated during the last 2-3 decades the Greater Athens area. The implications of these findings justify important concerns on reproductive health in some age-specific groups in the area of Greek capital.

1. Introduction

Human reproductive health has been declared as a basic right of mankind and its preservation is the main objective of various regulatory bodies operating both at global (e.g. the World Health Organization-WHO) and at regional level in many countries worldwide.

Over the last 20-30 years a wealth of information has been accumulated indicating that several parameters of reproductive health have been seriously compromised in industrialized societies. This deterioration has been attributed to a number of factors, the main being the changes observed in the environmental conditions prevailing in each particular area (Bostofte *et al.,* 1983; Bendvold, 1989; Zorn *et al.,* 1999; Kolstad *et al.,* 1999).

Moreover, great economic, social and cultural changes have drastically modified existing patterns for the expression of reproductive activity in these societies. The net outcome is a significant decline of procreation, as seen by the gradual dropping of birth rates, sometimes well below the replacement level.

In the course of our work in the field of the Endocrinology of Reproduction over more than a 30-year long period in the same Institution (Elena Venizelou Hospital in Athens), certain aspects of reproductive health have been investigated in a random fashion in large cohorts of people living in the Greater Athens area. The single common feature of all these studies was the finding of a significant deterioration of

important parameters related to reproductive life and activity of Athenian men and women during the last 3-4 decades.

The relevant studies performed and published over this period of time included:

- Observations on the declining trends of seminal parameters in Athenian men investigated for subfertility over a 17 years period.

- Investigation on the ratio of male to female babies born in this hospital over a 35 years period.

- Evidence to the effect that male newborn babies might have a higher mortality than their female counterparts.

- A high incidence of premature menopause in Athenian women who became recently postmenopausal.

- Very low awareness of Athenian men about the important consequences of gonadal ageing on their overall health and activity.

- Evidence that men with better awareness of health problems associated with ageing are better off than their less informed peers.

2. Reproductive health studies in Athenians

The aim of each one of these studies, the parameters examined, the findings and the main conclusions drawn are presented in some detail in the following separate entities:

2.1. Seminal volume and total sperm number trends in the Greater Athens area

The hypothesis that male reproductive capacity as expressed by a gradual decline of sperm numbers may have deteriorated in recent years was brought to wide attention by a meta-analysis study from the group of Skakkebaeck (Carlsen *et al.,* 1992). In this work, 61 studies published between 1938 and 1990, and representing populations of men with proven or unknown fertility from all geographical areas of the world, have been evaluated and a dramatic drop of mean sperm count was observed during the period studied (on average from 113.0×10^6/ml in 1940 to 66.0×10^6/ml in 1990). Following this publication, a large number of new studies appeared in the literature presenting data collected over prolonged periods of time

from fertile and infertile men or sperm bank donors and relevant for specific geographic areas and populations. In a number of studies, this decline has been confirmed (Auger *et al.,* 1995; Irvine *et al.,* 1996; Bonde *et al.,* 1998), whereas other investigations failed to substantiate it (Fisch *et al.,* 1996; Rasmussen *et al.,* 1997; Tortolero *et al.,* 1999). This discrepancy neither contradicts the validity of the meta-analysis observations nor is it the result of a distortion brought about by confounding the analysis factors. On the contrary, it is now accepted that differences in socioeconomic, environmental, geographic, dietary and life-style conditions may well account for the diversity of the data.

In Greece, a number of dramatic changes occurred during the last few decades enormously affecting everyday life and activity. These changes have been particularly evident in the Attica basin which houses approximately 40% of the country's total population. Therefore, one would expect that certain sensitive parameters of reproductive health of men living in the capital city and its surroundings may have felt the impact of such changes.

To investigate this assessment of the main sperm parameters was made in men living in the Greater Athens Area during this period (1977-93), who consulted for couple subfertility (Adamopoulos *et al.,* 1996). From a total of 23.850 cases in our records, a 10% sample was randomly selected for assessment for a possible changing trend over this period of time. The results of this study were viewed against a background of changes in some important air pollution indicators recorded over the same period in this particular geographic area. The main findings of the study are:

1. A declining trend of mean seminal volume was observed over the years of observation ($p < 0.05$). A seminal volume loss of 0.02 ml per year (error 5%) was estimated over the 17 years of the study.

2. A decrease in total sperm number was also observed during the study period, the trend being highly significant ($P<0.001$). Based on this observation an average annual drop of 3.0×10^6 of spermatozoa (error 5%) per ejaculate was estimated. An arbitrary distinction between samples with relatively low ($<120.0 \times 10^6$) and high ($240\text{-}400.0 \times 10^6$) total sperm number showed a marked decline in the latter subset, the frequency distribution for the latter dropping form 16.9±4.5% (1977) to 10.6±1.6 in the final year of the study ($p < 0.01$). Qualitative sperm parameters such as morphology and motility were not assessed in this study.

3. Over the same period of time, a significantly rising trend ($p < 0.01$ for each one) was observed for certain air pollution indicators such as NO, NO_2 and SO_2 (Table 38). On the other hand, an inverse trend was observed for air Pb content ($P<0.02$), a consequence attributed to the introduction of unleaded petrol in the country in the early to mid-1980's.

Table 38. Air and blood pollution indices in the Greater Athens Area (Adamopoulos *et al.,* 1996)

Air pollutants
- a rising trend for NO, NO_2 and SO_2 ($P<0.01$)
- initial phase increase, latter phase decline in Pb values
Blood Pb levels
- mean concentrations of 17.57 μg/dL for men
(upper limit 10.00 μg/dL, EEC instruction L 105/10-1977)

From the findings of this study it was concluded that a marked decline of seminal volume and total sperm number was observed in a large, racially homogenous, population of men at reproductive age living in the Greater Athens area who were assessed over a prolonged period of time. During the same period, a marked deterioration of the local environment was recorded. Obviously, any causative relationship between the biological and the environmental changes observed remains a matter of conjecture at present.

2.2. The changing ratio of male to female newborns

An important aspect of reproductive potential of a community is the relative size of the male population available for procreation at any given period during the reproductive life-span of the females in this society. In this context, it was important that a number of recent studies have demonstrated a statistically significant decline in the male to female newborn ratios (Feitosa and Krieger, 1992; Dickinson and Parker, 1996; Moller, 1996; Van der Pal-de Bruin *et al.,* 1997; Allan *et al.,* 1997).

To assess our domestic situation, data from the records of our institution that is a busy, inner city, maternity hospital, were evaluated with regard to the sex distribution of the newborn babies over the last three and a half decades (Adamopoulos, 2000).

During the years 1969 to 2003, a total of 221.799 live baby deliveries were recorded in the hospital's archives. During that period, the absolute number of male newborns showed a gradual reduction. Of greater importance however, was the declining trend in the male to female newborns ratio. This ratio, universally found as greater than one, showed a drop from 1.09 in 1969 to 1.04 in the final year (0.95% drop, $p<0.01$). Therefore, it appears that a marked decrease has occurred not only in the absolute but also in the relative number of newborn males as compared to female babies born in that 35-year period and in this particular population (Figure 18). This finding is in accordance with observations from such diverse populations as the British (Dickinson and Parker, 1996), the North Americans (Allan *et al.,* 1997) or the Latin Americans (Feitosa and Krieger, 1992) which were all statistically significant although tiny in magnitude. Moreover, it may be relevant to the deterioration of pollution indicators in the area, shown in section 2.1, and the generally accepted interpretation that this sex ratio reflects the presence of mutagens in the environment.

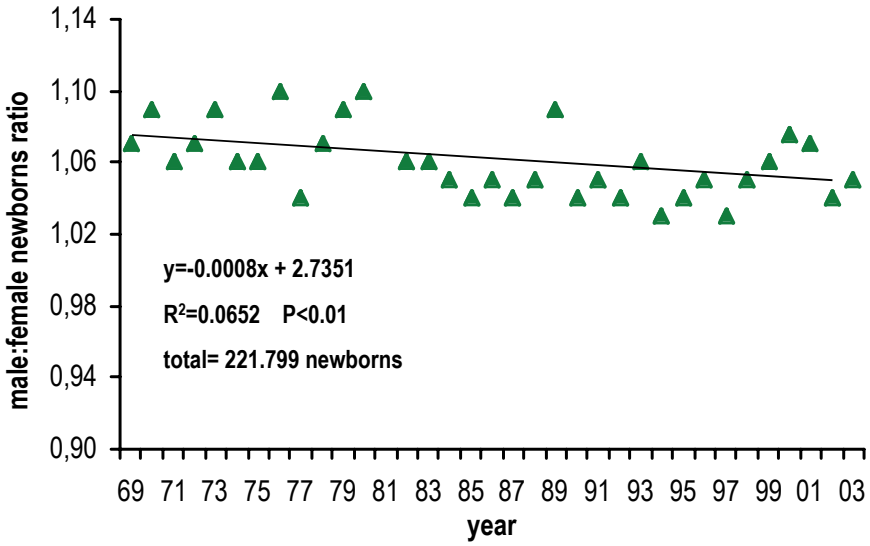

Figure 18. Ratio of male to female newborns in Elena Venizelou Maternity Hospital in Athens (Adamopoulos, 2000)

2.3. Higher perinatal risk factors for male newborns

Male sex has been earmarked as a risk factor for fitness and survival in recent studies (Cooperstock and Cambell, 1996; Cooperstock *et al.,* 1998). Indeed, the proportion of male pre-term infants is higher than that in of females, and in white

singleton births less than 37 weeks of gestation it was estimated to be 55% in the U.S.

Observations, from the same centre as the one providing data for the previous work (see 2.2.) were recently presented by Savoglou *et al.* (2001, 2002). The sex specific differences in the delivery profile, the outcome of delivery, the anthropometric characteristics and the parameters related to perinatal risk factors were assessed in the total number of newborn babies for one year (1998). Out of a total of 4969 neonates there were 2554 (51.5%) boys and 2415 (48.5%) girls. The sex prematurity ratio was similar to that of absolute numbers ratio (male to female), as were other parameters such as the mode of delivery, the perinatal mortality, etc., in the two sexes (Table 39). However, when it came to risk factors, it was noted that perinatal care was necessary for 538 out of the 2554 newborn males, the corresponding figure being 472 for the 2415 newborn females. Moreover, mortality during intensive care therapy was 4.3% (n = 23) for the male and 3.2% (n = 15) for the female newborns.

Table 39. Some relevant data from preproductive health problems (single year – 1998- data) (Savoglou *et al*., 2001)

Parameters	Male newborns		Female newborns		m:f ratio
	n	%	n	%	
Total number	2554 - 51.5		2415 - 48.5		1.057
Premature <37w	229 - 51.7		218 - 48.3		1.050
Hospitalisation	538 - 54.3		472 - 45.7		1.139
Mortality	23 - 60.5		15 – 39.5		1.533

Overall, these limited data from our Centre are in agreement with the findings of other investigators in the field (Zeitlin *et al.,* 2002; Mace *et al.,* 2003; Cagnacci *et al.,* 2003) and most certainly raise some important questions related to the cause of male newborns vulnerability. One may assume that a number of factors at play during gestation may be instrumental in bringing about this biologically inferior male offspring condition. Work on this topic is now being extended to include more parameters and years of data collection from our Hospital.

2.4. High incidence of spontaneous or induced menopause

The mean age of natural menopause is relatively stable in Western Societies, at an average age of 50-51 years. On the other hand, spontaneous permanent cessation of menstruation with hypergonadotropism before the age of 40 years (WHO 1996), has

been estimated to approximate 1% with small, although significant variations related to race or ethnicity may be found (Farrell, 2002; Luborsky, 2003). However, this may have been an underestimation if the hypothesis that up to 10% of the women of general population who became postmenopausal by the age of 45 may have experienced an accelerated decline of their fertility before the age of 32 holds true (Nikolaou and Templeton, 2004). Regarding the situation in Greece there has not been recent and detailed study on this topic except an early publication on the mean age at menopause for Athenians, more than a quarter of a century ago (Batrinos *et al.,* 1979). Since, a number of great changes in dietary, life-style and health considerations took place in our society during that period, it was essential that a fresh and more detailed investigation into the age at menopause and its different subtypes had to be undertaken.

To this end, the age at menopause and the prevalence of its different subtypes in contemporary Greek women were investigated in a cohort of subjects who became postmenopausal during the last 1 to 5 years. This time-range was deliberately chosen since older women are often vague about the exact time of their last menstrual period (Adamopoulos *et al.,* 2002). The overall clinical material screened for recent menopause was a population of 15.000 women attending the Endocrine Clinic of this hospital and the number found was 1.747 women, 21 to 66 years old at the time of their menopause. To offset any confounding factors due to the nature of the clinic, an additional group of 4.000 women attending the Internal Medicine and General Surgery Departments was also screened and 438 women, aged 25 to 68, were found to have recently become postmenopaused. The core findings of the study in relation to reproductive health issues are (Table 40):

a) A total of 10.5% of the postmenopausal women (7.1% spontaneous, 3.4% induced) had their menstrual periods terminated before the age of 40 and they should be considered as premature menopause cases (WHO, 1996). This incidence is similar to the incidence observed in the control group.

b) A high incidence of spontaneous or induced menopause (11.7%) after the age of 40 was also observed both in the study and in the control group.

c) No significant relationships between the various reproductive life events and age at menopause were found (Table 41). In the premature menopause group, a significant relationship with age at menopause and smoking was noted ($P<0.05$); furthermore the age at menopause for smokers was lower (47.7±2.7) than that of non-smokers (49.0±2.7) but the difference did not reach statistical significance. However, it should be mentioned that the smokers group was very small since this habit was taken up by young Greek women only during the last

20 years or so therefore, its impact on the age of menopause has not been manifested as yet.

d) In total, an alarming figure of 22.2% of the women in this sample had their hormonal activity terminated well in advance of the mean age at natural menopause for women living in the same area estimated as 48.7±3.8 years (mean±SD).

Table 40. Incidence of premature menopause in Greek women (Adamopoulos *et al.,* 2002)

	Total	Spontaneous	Induced
1. Premature menopause – incidence % (<40 years)			
Study population	10.5	7.1	3.4
Control population	10.5	5.9	4.6
2. Premature menopause – incidence% (>40 years)			
Study population	11.7	6.7	5.0
Control population	19.6	9.3	10.3

Table 41. Relationships examined between various reproductive life events and age at menopause (Adamopoulos *et al.,* 2002)

age at menopause (whole group)	versus	type of cycle no of pregnancies no of abortions no of babies delivered lactation tract surgery alcohol/smoking		N.S.
premature menopause	versus	smoking	r = 0.231 p<0.05	
smokers (47.7±2.7)	versus	age at menopause		N.S.
non-smokers (49.0±2.7)	versus			

Overall, it appears that a considerable number of Greek women enter menopause at a relatively young age. Moreover, this proportion is considerably higher than the 1.0% observed in American women (Coulan *et al.,* 1988; Luborsky *et al.,* 2003). In

fact, the total of spontaneous (7.1%) and induced (3.4%) premature menopause incidence (10.5%) has never been encountered in any other study and its magnitude reaches the limits of an epidemic for this population. A number of factors may be involved in bringing about an early permanent ovarian decline these including chromosomal abnormalities, autoimmunity, changed life-style conditions, etc. Certainly, life style conditions and dietary habits have tremendously changed over the last years. Moreover, the introduction of smoking by young Greek women has been a noticeable trend in the women's sexual liberation generations in the late sixties and seventies. Indeed, cigarette smoking has been closely related with the age at menopause in different populations and with the age at premature menopause in our population (Adamopoulos *et al.,* 2002). This factor, among others, may have been of paramount importance in the early termination of reproductive life in this group of women.

The wider implications of our findings and the health concerns are twofold: Firstly, the reproductive capacity in a large part of this female population was compromised very early in life with most of those women being unaware and totally unprepared for an early change of life. This decline of reproductive potential is not only defined by the early age of menopause but mostly by an increasing reduction of fecundability which occurs some 10-12 years prior to the last menstrual period. Therefore, even women in their twenties destined to have a premature menopause, may experience conception difficulties.

Secondly, this state of severe oestrogen depletion defined a relatively large group of women who not only were deprived of their ability to procreate but also had the need for hormone replacement therapy at a relatively young age and for a prolonged period of time. This need has apparent personal, social and economical implications and create a burden on the Health System of the country.

2.5. Low awareness of the consequences of gonadal ageing

Prevention or early treatment of reproductive and related disorders is closely associated with early awareness of the problem and its consequences. A lack of awareness may be an important factor in bringing about reproductive health problems. A typical example of low public awareness for some ageing-related problems has been demonstrated in a sample of Athenians. A special investigation was designed to explore the attitudes of a subset of the male population of Athens on issues related to andropenia, which is the term describing gonadal failure in ageing

men (Adamopoulos, 1998), its consequences and the perception that some kind of remedy as hormone substitution therapy might be of help.

To this end, a total of 160 men, 50-75 years of age were randomly selected and in a proportional fashion from a population of 366.860 men of that age-range living in Athens (Adamopoulos *et al.,* 1999). The study intended to chart and characterize the prevailing views on ageing men's declining gonadal function and was performed employing the method of face to face interviews with semi-structured questionnaires. Of the findings of the study, two were closely related to prevention or early detection of a reproductive health problem.

a) Awareness of the problem and its consequences: Only 27% of the group knew something about health problems associated with a gonadal decline in ageing men, its general consequences and its related terminology. Out of this small group, 79% came from the upper socioeconomic class, the source of information being news media (60%), word from wives and partners particularly those on hormone replacement treatment (37%), and information gathered at the work-place (3%).

b) Need for investigation of the problem and treatment: Of the men interviewed, relatively few thought that a visit to a physician was important (31%) or very necessary (15%) and of these, 54% came from the 'over the age of 60' group.

Table 42. Awareness of ageing man's gonadal decline (Adamopoulos *et al.,* 1999)

a) 27% of the sample had some information
b) 73% had no idea of the problem or its consequences
c) of those informed:
- 79% came from high socioeconomic class
- 60% were informed by the media
- 37% were informed by friends or mates
- 3% were informed at their work-place
- 46% thought medical advise necessary

As, it is evident not only a large proportion of ageing men were unaware of problems related to their reproductive health at this stage of life, but their perception of the need for investigation and therapy was also very low. In this context, prevention or early treatment for their relevant health problems was not a popular option.

2.6. Evidence for the beneficial effect of problem awareness

To test the possibility that a better awareness of a reproductive health problem might be associated with better overall health conditions, a study was designed with assessment of a group of ageing hospital doctors, with a high degree of problem awareness, in comparison with a group of administrative personnel of similar age with a lower awareness standard (Nicopoulou and Adamopoulos, 2001).

In both groups, assessment of physical, mental and sexual activity was made using properly validated questionnaires whereas an endocrine evaluation included measurements of FSH, LH, PRL, TSH, T and SHBG. The main findings of the study are shown below (Table 43).

Table 43. Comparison of reproductive health indicators between ageing doctors and admistrative personnel (Nicopoulou and Adamopoulos, 2002)

	Ageing doctors
(a)	- better physical activity (+7.8%)
	- better sexual activity (+5.2%)
	- less symptoms of andropenia (-10.4%)
(b)	- higher T:LH ratio ($P<0.001$)
	- lower SHBG concentration ($P<0.01$)

It is noted that physical, mental and sexual function as well as androgen activity were significantly better in the ageing doctors as compared with the group of the administrative personnel of similar age. This may have been the result of a better health education and self-protection, healthier dietary and life-style habits and even better financial condition. All these factors may offset the stressful working conditions in the doctors group.

3. Discussion

In this series of studies on reproductive health issues, the special characteristics are the large number of data, the ethnic homogeneity of the population studied, and the fact that the common place of work and life of the people studied was the Greater Athens area. The main findings are in line, in most cases, with observations from similar studies by other groups in different populations and geographic areas. In the studies described in this paper the overall picture is indicative of an important

deleterious effect observed over the years in aspects of reproductive health in Athenians (Table 44).

Significantly declining trends in important sperm parameters have been demonstrated in men assessed over a prolonged period of time. This trend is more evident in men with high sperm count. Similar trends in sperm parameters have been observed in a number of studies (Auger *et al.,* 1995; Irvine *et al.,* 1996; Bonde *et al.,* 1998) as well as in a meta-analysis (Carlsen *et al.,* 1992), but any speculation as to the causative factors involved was not properly supported by the design of the studies.

Table 44. Special characteristics of the studies' reproductive health

a.	Ethic homogeneity of the samples investigated
b.	Large number of data analysed covering extensive periods of time
c.	Same geographical area and environmental conditions
d.	A steady increase in environmental pollution indicators over the last years
e.	A deterioration in all aspects of reproductive health studied

At the same time a declining trend in the male to female newborn baby ratios has been demonstrated in a large number of deliveries recorded over a 35-year period. This change has not been restricted to our geographic area and probably signifies a broader biological change. Moreover, this finding was further expanded by the suggestion that male newborns might be more susceptible to perinatal risk factors than females, although this was not statistically significant in this particular study. However, it merits more extensive research, since there is corroborative evidence coming from other works.

Regarding women, an alarmingly high incidence of premature menopause cases has been observed in a large cohort of Greek women. The implications of this finding in relationship to the reduced reproductive capacity of these women and/or the need for long term hormone replacement treatment are quite obvious. Moreover it merits special attention since it is unique among European populations and it reaches the dimensions of an epidemic.

Finally, a gross insufficiency of public awareness on reproductive health issues has been demonstrated in ageing Athenian men who, in their great majority, knew very little about the consequences of gonadal decline on their overall health and activity.

Moreover, there was almost a total lack of information regarding investigative procedures and means to combat the problem.

These findings became more relevant and meaningful with a finding of a better overall physical, mental, sexual and biochemical condition in properly informed men, such as medical doctors.

From this composite picture it becomes evident that there is a need for a proper education of the general public on reproductive health issues which should be seen as a social necessity and a task for health professionals in the years to come. Finally, recognizing and treating the consequences is only part of the problem. Equally important is the identification of any causative factors, for reproductive health problems. Therefore, although public awareness and therapeutic measures are important, prevention emerges as the ultimate task. In this context, the issue becomes broad and requires the intervention of policy makers and the general public.

By and large, it appears that a number of problems related to the reproductive health of contemporary Athenians has gradually emerged in recent years and while their cause has not been clearly established at present, their impact is well documented and felt. Whether, multiple and different factors or the common overall adverse environmental milieu or both were related to any or all of the relevant aspects of severed reproductive health is a matter of conjecture at present. However, it is clear, that the overall pattern of reproductive health problems occurring in this geographic area is more or less similar to that observed in other parts of the industrialized world.

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ENVIRONMENT AND REPRODUCTIVE HEALTH IN BULGARIA

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Summary

Reproductive health indicators are chosen for assessment of reproduction problems in Bulgaria by using official medical statistical information. Based on National registers a rough picture of the environmental pollution in Bulgaria is depicted by considering selected persistent organic pollutants (DDT, dioxins and furans) and some heavy metals (Pb and Cd), suspected to be reproductive toxins. Assessment was done on the basis of trends in birth and death rates, and some disruptions in reproductive functions. Significant and persistent reduction of the population, as well as a negative population growth, are established. Comparatively high male and female infertility was recorded. Prenatal and postnatal developmental problems were detected. Slight increase of the stillbirth rates and lack of statistical difference of the spontaneous abortion rates during the last years were established. Increase of mortality rate due to low birth weight and preterm delivery was noticed till 1991 with a slight reduction thereafter. Trends of reduction in the infant mortality rates were established, but still were higher than the other European countries.

Attempts to localize reproductive health problems to 'hot spots' of environmental pollution in Bulgaria were performed. It is concluded that the reproductive problems in Bulgaria are a combination of socio-economic, environmental and genetic factors.

1. Introduction

We entered the new millennium with an enormous increase of anthropogenic chemicals and with widespread food, land, and water and air pollution. Billions of

P. Nicolopoulou-Stamati et al. (eds.), Reproductive Health and the Environment, 353–371.

tons of man-made chemicals have been manufactured and released into the environment. Most of them are known to be toxic to laboratory animals, wildlife and humans. In addition, various human activities lead to elevated levels of hazardous metals, including cadmium, lead, and mercury in the environment.

There is evidence, for reproductive and developmental effects of parental exposure to environmental pollutants, or pesticides, solvents, drugs etc. (Garcia, 2001). Pesticides constitute a group of wide number of compounds from very different chemical families, with a variety of toxicological characteristics. As a representative of the group of organochlorines, DDT is one of the most dangerous insecticides, which is included in the group of the forbidden for use persistent organic pollutants.

This paper presents an over all picture of the environmental pollution of the country and focuses on over all selected persistent organic pollutants (DDT, dioxins and furans) and the heavy metals Pb and Cd, suspected to be reproductive toxins. In addition, statistical data for some health indicators related to reproduction are presented.

2. Environmental pollution

Persistent organic pollutants (POPs) is a class of chemical that remain in the environment for a long time, resist degradation, are toxic and can travel long distances. In 1995 UNEP developed a “short list” of 12 POPs, which pose threats to human health and environment. It includes 9 pesticides, polychlorinated biphenyls (PCBs) and dioxins and furans. On the basis of the information on their properties and behavior of POPs, the Stockholm Convention was adopted in May 2001 by 92 countries. Parties to the Convention agreed that international measures should be taken for risk reduction for human health and the environment. The Rotterdam Convention, concerning the international trade of some dangerous chemicals and pesticides, and the Basel Convention, for the control of trans-boundary movement of hazardous wastes, are the other international provisions adopted by Bulgaria. As a party of the conventions, Bulgaria agreed to perform an inventory of the POPs – pesticides – total amounts stored, their distribution in water, soil, food and emissions of dioxins and furans.

Pesticides are not produced in Bulgaria. They have been imported from the Soviet Union. In 1971 import of DDT in Bulgaria was forbidden, and by 1976 it was out of use in the country. An inventory, carried out in 1996 in correspondence with Directive 67/548/EEC and the Annex 5 requirement to determine amounts of

"recently available chemicals", established that 35 tons of DDT are stored in Bulgaria with part of them being deposited in ruined, unprotected stores.

To destroy pesticides banned from use, including 35 tons of DDT, a contract between the Ministries of Environment of Bulgaria and the Netherlands was signed, and the Dutch firm AVR-International was employed to incinerate the chemicals. The pesticides were identified, quantified and repackaged in suitable UN approved and labeled containers, then transported under conditions that met legal requirements of both the Netherlands law and the Basel convention. As a result, the most dangerous chemicals from six storage areas in Bulgaria were eliminated.

Low concentrations of POP are still found in Bulgaria in the soil, the ground water and food. These residual POP concentrations are primarily due to persistence in the environment, the continued small-scale use, either illegal or through ignorance, by individual growers, as well as by trans-boundary movement by air, by water and by importation in foodstuffs.

The content of pesticides in the water is monitored by the National Automated System for Environmental Monitoring (NASEM) and in the soil by the Regional inspectorates of the Ministry of environment and water. Table 45 presents a comparison between the mean environmental concentrations of DDT in Bulgaria and the standardized concentrations. At the end of the nineties, the mean annual concentrations of DDT in ground water exceeded the pollution threshold (PT) of 0.1 μg/l. By 2000, the mean annual concentration had dropped below the ecological threshold (ET) of 0.01 μg/l. The mean annual concentrations of DDT measured in soil in 1997 and 1998 were higher than the protective concentration (PC) of 0.3 mg/l. Concentrations dropped below the PC in 1999 and 2000, but in 2001 they exceeded it again. As could be expected, the mean concentrations of DDT in the air in rural areas are up to ten times higher than in urban areas.

There are limited data on the concentrations of DDT in basic foodstuffs. These show that in 1987 and 1989 concentrations in meat were substantially above the MAC, but that wheat, potatoes and milk contained DDT in concentrations below the MAC for those products.

Polychlorinated dibenzodioxins (DBD) and dibenzofurans (DBF) are by-products of combustion, especially of plastics, of chlorinated products manufacture, and paper production. Dioxins and furans are a group of chemicals without practical use, and are therefore not produced on purpose, but only as by-products of other synthetic processes. Dioxins and furans are formed during the thermal treatment of organic

chlorine-containing compounds, and as by-products during the synthesis of chlorine-containing chemicals such as chlorinated phenols or herbicides.

Table 45. Environmental concentrations of selected POPs in Bulgaria

Concentrations	Chemical			
	DDT (total)		Dioxins and Furans	
	Standard	Status	Standard	Status
Ground water	ET = 0.01 µg/l PT = 0.1 µg/l	Mean annual conc.,µg/l 1998 - 0.1171 1999 - 0.4463 2000 - 0.0058 2001 - 0.0003 2002 - 0.0034	SAE = 0.5 ng I-TEQ/l waste water MAC_{PAH} in drinking water = 0.10 µg/l	No monitoring
Soil	PC=0.3 mg/kg DS MAC= 1.5 mg/kg DS CLI=4 mg/kg DS; US EPA AHR-2-3ppm	Mean annual. conc., µg/kg 1997 - 714.7 1998 - 318.8 1999 - 113.6 2000 - 120.1 2001 - 316.4	BL_{PAH} = 0.15 mg/kg PC_{PAH} = 0.4 mg/kg MAC_{PAH} = 4 mg/kg	No monitoring
Air	EC standard 20 pg/m^3	Rural areas = 1-22.10^{-6} ng/m^3 Others-1 = 2.3.10^{-6} ng/m^3	SAE_{DW} = 0.1 ng I-TEQ/m^3	No monitoring
Food	MAC_{meat} =100 µg/kg MAC_{potato} =100 µg/kg MAC_{wheat} =200 µg/kg MAC_{milk} =1000 µg/kg fat	Meat: 1987-4328µg/kg 1989-1100 µg/kg 1991: Potatos 92 µg/kg Wheat 17 µg/kg 1995: Milk – to 387 µg/kg	EC recommended levels for food and forage	No monitoring

Ecological threshold – ET; Pollution threshold - PT

Protective concentration – PC; Maximal allowable concentration – MAC; Concentration level for intervention – CLI; Dry soil - DS

Accept health risk –AHR

Standard for allowable emissions of incineration of domestic waste – SAE_{DW}

Background level of polycyclic aromatic hydrocarbons (PAH)- BLPAH

There is no monitoring system for dioxins and furans in air, water, soil or food in Bulgaria. Standards for maximum allowable concentrations of dioxins and furans in the ambient air, surface water, drinking water and soil are not adopted, but the Bulgarian standard for allowable emissions from incinerators of domestic waste is published by the Ordinance No11/1998 (SG 152/1998). In addition, standards for contamination of drinking water and soil with polycyclic aromatic hydrocarbons (PAH) have been adopted.

By using the emission model CORINAIR-94, national inventory of the emissions from different sectors in Bulgaria has been carried out. The calculations used in the emission inventory have been adapted to the specific national features of technologies, installations, raw materials and fuels. Table 46 shows total calculated emissions of dioxins and furans from different sources

Table 46. Emissions of dioxins and furans emitted in 2000 into the atmosphere of Bulgaria according to the type of the source (Sokolovski and Dombalov, 2005)

Source	Amount, g/year	% of the total emissions
Total emissions	232.5	100
Burning of fuels in energy production	111.6	48
Burning of fuels in non energy sectors	58.1	25
Industrial processes	20.9	9
Industrial combustion processes	16.3	7
Waste treatment and disposal	9.3	4
Mobile motor means and appliances	9.3	4
Road transport	7.0	3

Burning of fuels in both the energy and non-energy sectors accounts for 73 percent of the pollution, with thermal electrical and metallurgical plants being the main sources emission.

The mean annual emissions of dioxins and furans from Bulgaria are compared with ten other countries in Table 47. Bulgaria emits the sixth highest total amount of dioxins and furans, but emits the third highest amount of these products after Belgium and Japan, when annual emissions per unit area are compared. It is noteworthy that it has almost the same emissions as the USA and UK, which are more industrialized countries.

Table 47. Annual amounts of dioxins and furans emitted in different countries (1995) (UNEP, 2001)

Country	Annual emissions, g/I-TEQ/year	Average annual emissions for unit area, g/sq. km/year
Japan	3984	0.0102
USA	2744	0.0050
France	873	0.0017
Belgium	661	0.0215
UK	569	0.0020
Bulgaria	456	0.0038
Germany	334	0.0010
Austria	29	0.0007
Switzerland	181	0.0040
Australia	150	0.0001
Denmark	39	0.0008

The most polluted countries in Europe, with mean ambient air concentrations exceeding 20 fg/m^3, are the Czech Republic (12 – 52 fg/m^3), Belgium (10 – 27 fg/m^3), and Slovakia (10 - 28 fg/m^3) (UNEP, 2002).

There has been a consistent reduction in total emissions of dioxins and furans into the atmosphere of Bulgaria between 1990 – 2002 (Figure 19) with the 2002 emissions calculated as about 60 per cent of that in 1990. 95% of dioxins and furans enter into the human body by food, mainly by meat, eggs, milk and milk products, fish etc. They accumulate in different parts of the body. It is supposed that they are eliminated in the form of water soluble biotransformed sulfates. Women release dioxins through the caul during pregnancy and through the mother's milk during lactation. Bulgaria is far below countries such as the Netherlands, according to the concentration of PCDD and PCBs in the human milk (with 6.14 pg WHO-TEQ/g fat against 18.27 pg WHO-TEQ/g fat for the Netherlands (MoEW, 2004).

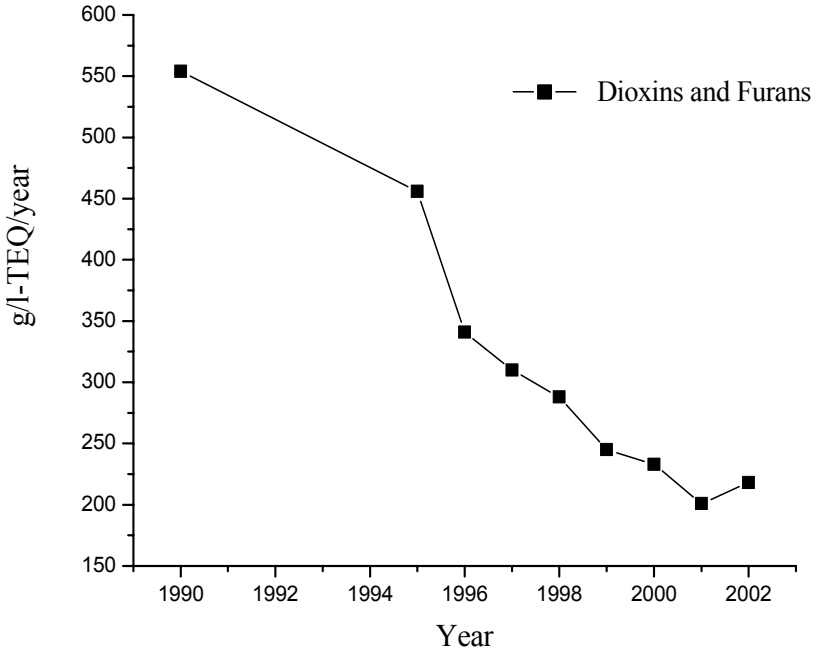

Figure 19. Annual emissions of dioxins and furans into the atmosphere of Bulgaria (MoEW, 2004)

The soils polluted with heavy metals in Bulgaria are studied and mapped in details (MoEW, 1999). Total area of the land polluted with heavy metals over the standards is 43,660 ha, or 0.7 percent of the agricultural land. Pollution is localized in areas where mining and extractive metallurgy are developed, near factories from the ferrous and non-ferrous metallurgy, along the high ways and main roads. In Table 48 polluted “hot spots” with maximal metal concentrations over norms are shown. Pollution is expressed by toxicity factors (Kt) showing the degree of exceeding standards.

The main pollution of the soil is due to old environmental burdens caused by industrial waste water and tailing ponds for industrial wastes. It is caused by poor environmental exploitation, lack of effective installations for purification of wastes and low environmental control in the past.

Table 48. "Hot spots" with metal concentrations over standards (MoEW, 1999)

Localization	Source of pollution	Kt $_{Pb}$	Kt $_{Cd}$
Yana village-500 m far from "Kremikovtci"- near Sofia	Ferrous metallurgy	3.5	-
500 m far from the plant for nonferrous metals-Plovdiv	Nonferrous metallurgy	26.4	13.9
Home yards near metallurgical plant "Eliseina"	Extractive metallurgy	2 – 8 (Acid soil)	1 – 2 (Acid soil)
Ore dressing factories in the region of Smolyan	Mining	1.3 - 1.6	0.5 – 1.0
Village "Novi Iskar" near Sofia	Industrial zone	4.4	-
Region of "Chiprovtci" near Montana	Mining	1.5 – 4.9	-
"Chepelarska" river near Plovdiv	Highway	1.6	1.5
Highway "Trakia"	Highway	1.2 – 1.6	-
Sofia – Pernik – Kulata	First class road	2.8-2.9	-

3. General demographic data

During the last 10 –15 years unfavorable trends in the demographic processes in Bulgaria were established (Table 49).

During the whole period the population growth rate is increasingly negative. The highest negative value of the growth rate is recorded in 1997. This pattern of negative growth in population is influenced both by an extremely low birth rate and a relatively high death rate in addition to a comparatively high rate of emigration. The population decrease that began in 1990 continues to the present with most Bulgarians living in urban areas. A limited in the increase rural population is noticed during period 1992 – 1998, probably because of the increase of unemployment in the cities. Females predominate, constituting a little over fifty percent of the population. Women in the age group of forty predominate over males as well, which means that the male's death rate is higher. There is a slow, but noticeable, constant decrease of the percentage of men in the Bulgarian population.

Table 49. General demographic data in Bulgaria (NSI, 1991 –2003)

Year	Population			Male/Female Ratio	Growth rate per 1000 people		
	Total	In urban areas, (%)	In rural areas (%)		Total	In urban areas	In rural areas
1990	8669285	68.3	31.7	49.2/50.8	-0.4	3.2	-7.7
1991	8603219	68.2	31.8	49.3/50.7	-1.6	1.5	-8.0
1992	8584800	67.2	32.8	49.1/50.9	-2.2	0.9	-8.5
1993	8459763	67.6	32.4	49.,1/50.9	-2.9	0.2	-9.5
1994	8427418	67.8	32.2	49.0/51.0	-3.8	-0.7	-10.4
1995	8384715	67.,8	32.2	48.9/51.1	-5.0	-1.9	-11.9
1996	8340936	67.6	32.4	48.9/51.1	-5.4	-2.0	-12.3
1997	8283200	67.,7	32.3	48.9/51.2	-7.0	-3.5	-13.9
1998	8230371	68.0	32.0	48.8/51.2	-6.4	-3.0	-13.6
1999	8190876	68.1	31.9	48.7/51.3	-4.8	-1.8	-11.1
2000	8149468	68.4	31.6	48.7/51.3	-5.1	-2.0	-11.5
2001	7928901	69.0	31.0	48.7/51.3	-5.6	-2.6	-12.3
2002	7845841	69.6	30.4	48.6/51.4	-5.8	-3.0	-12.5

The negative growth rate is much higher in the rural areas than in the urban ones. Even though in urban areas the growth rate has positive values till 1993, it is strongly negative, even in 1990, in the rural areas.

4. Reproductive health

To assess the reproductive health status in Bulgaria, official medical statistics were used. Assessment is done on the basis of trends in birth and death rates, and chafes of the reproductive functions.

4.1. Birth, fertility and death rates

In 2002, 67038 babies were born in Bulgaria, 1681 fewer than in 2001. Trends in birth statistics are shown in Table 50. There has been an approximately ten per cent reduction of crude birth rate between 1994 and 2002.

Table 50. Birth and fertility rates (NSI, 1995 -2003)

Year	Crude birth rate per 1000 person, ‰	Total fertility rate per woman	Crude fertility rate per 1000 women at fertile age, ‰	Sex ratio at birth (male/female)
1994	9.4	1.37	38.7	1.04
1995	8.6	1.23	35.0	1.05
1996	8.6	1.24	35.2	1.06
1997	7.7	1.09	31.4	1.08
1998	7.9	1.11	32.2	1.06
1999	8.8	1.23	35.8	1.05
2000	9.0	1.27	36.7	1.05
2001	8.6	1.24	35.7	1.06
2002	8.5	d.n.a	d.n.a	d.n.a

d.n.a. – data not available

In parallel with the reduction of the birth rate, the total fertility rate per woman and the crude fertility rate have decreased. All three indicators were minimal in 1997 and they have slightly increased in subsequent years, till 2000.

Many factors exist that influence the birth rate. Among them are the socio-economic factors specific for the country, the general world trends of reduction in marriage, the increasing age for women giving birth to their first child, reduction of the number of children in the family etc. As well as socio-economic effects, the health status of the people in their reproductive age play is essential in birth reduction. The reproductive ability of both men and women is significantly affected by their life style, including such variables as diet, tobacco, alcohol consumption and occupational conditions. Environmental pollution in the working place, at home and the general environment has significant effects on reproduction. Reproductive failure is of serious concern, both for families and society.

The EU Directive on dangerous substances 67/548/EEC and its amendments identifies “toxic to reproduction” as one of the risks. According to this, toxicity to reproduction concerns two areas of toxicity in addition to effects on lactation:

- effects on male or female fertility;

- developmental toxicity.

Developmental toxicity includes effects that interfere with normal development, both prenatally or postnatally, that is before and after birth. These effects can be manifested as reduced body weight, developmental retardation, organ toxicity, death, abortion, structural defects (teratogenic effects), functional defects and impaired postnatal mental or physical development up to, and including, normal pubertal development.

Substances toxic to reproduction include those that have effects on lactation causing harm to breast-fed babies.

The Inventory of Fertility in Bulgaria shows that in 2004 the numbers of the infertile women and men were approximately equal at 200 000 each. The annual number of the babies conceived "*in vitro*" is 200 (Bulgarian Parliament, 2004).

Over the past few years, public awareness of the possible risk to the developing embryo and fetus following exposures to environmental agents increased. Reproductive hazards of environmental agents have been assessed by monitoring the rate of stillbirths, spontaneous abortions, congenital abnormalities, prenatal complications, mortality and, occasionally, postnatal growth or mortality (Ornoy, 2000). Some studies have found an increased risk of fetal death of both spontaneous abortions and stillbirths, related to pesticides usage (Saxema *et al.,* 1980; Rosenberg *et al.,* 1987; Mc Donald *et al.,* 1988; Zhang *et al.,* 1992; Nurminen, 1995). The authors consider risks of fetal deaths associated with general pesticides exposure and with exposure due to maternal agricultural work. In 2000, a Europe-wide Workshop was held in Sofia, supported by the Human Potential Programme at the European Commission. The purpose was to review the present state of knowledge concerning the possible impact of pesticides in current use on human reproductive and developmental health, and to encourage future research.

Statistical data for Bulgaria for the period 1994 - 2002 show a slight increase of the stillbirth rates, and a lack of statistical difference of the spontaneous abortion rates – between 4.7‰ in 1997 and 5.1‰ in 2001. For the same period, the total number of abortions per 100 pregnancies finished by delivery (or abortions/births ratio) has decreased by 45 per cent, due to the changing of socio-economic conditions (table 52).

The increasing number of chemicals usage in the environment might be responsible for the decline of the male/female ratio among newborns (Mocarelli *et al.,* 1996). Data in Table 50 show that the ratio is relatively constant, being between 1.04 and 1.08 over the period considered. Bulgaria has a lower ratio than the Russian average

of 1.2 (Revich *et al.,* 2001) and this value has remained the same for Bulgaria since the beginning of the century, with values of 1.06 in 1920; 1.06 in 1930 and even in 1976, when DDT was broadly used as insecticide: the ratio was 1.06.

Table 51. Trends in stillbirth rates and abortion rates in Bulgaria (NSI, 2003)

Year	Stillbirths rate per 1000 births, ‰	Abortion rate per1000 women at fertile age, ‰	Abortions/ births ratio, %	Spontaneous abortions, as part of all abortions, %
1994	6.2	47.5	122.1	d.n.a.
1995	6.4	47.2	134.1	d.n.a.
1996	7.6	48.1	128.6	d.n.a.
1997	7.5	43.1	136.0	11.0
1998	7.4	39.3	121.2	13.2
1999	7.4	35.8	99.4	15.4
2000	7.5	30.6	82.7	17.3
2001	7.3	26.8	74.5	19.2
2002	8.0	d.n.a.	d.n.a.	d.n.a.

d.n.a. – data not available

Mortality rates for Bulgaria are shown in Table 51. A small increase in crude death rates for all ages is noticed. In general, for the whole period, infant mortality rates in rural areas are higher than for the urban areas. As could be seen, 1997 is critical, with the highest mortality rates both in urban and rural areas. Even in 2002, the infant mortality in Bulgaria is very high, and is comparable only with the infant mortality rate in Russia. It is much higher than the rates in countries such as Austria (4.06 ‰), UK (5.23‰) or Norway (3.36 ‰). It is even higher than Albania (10.44 ‰) or Macedonia (10.19‰). It seems unlikely that data could be explained by environmental factors. More likely reasons are lower standards of medical care, and other socio-economic problems in the rural areas associated with general economic difficulties in 1997. Most infant deaths are primarily associated with complications of prenatal period, and to a lesser extent with congenital anomalies (Table 53). Again, these data show that 1997 is critical with respect to mortality rates. For all indicators, general trends of reduction are established.

In the literature low birth weight and preterm delivery are used as indicators of environmental pollution, e.g. Cd in high doses during the pregnancy penetrates trough the caul, disturbs embryonic development causing low body weight at birth. It could be a contributing factor for spontaneous abortions as well. Table 52 and

Table 53 present some statistical data for Bulgaria. The trend of increase in the complex indicator exists to 1991, followed by reduction in 1994. If environmental etiology of the incidents is accepted, these data could be explained by the reduction of environmental pollution in Bulgaria due to the decreased industrial production and use of pesticides after 1990, as well as the emissions linked with them.

Table 52. Crude mortality rate and infant mortality rate (NSI, 2003)

Year	Crude death rate per 1000 people, ‰	Infant mortality rate per 1000 live births, ‰		
		Total	Urban areas	Rural areas
1994	13.2	16.3	15.2	18.9
1995	13.6	14.8	14.0	16.7
1996	14.0	15.6	14.8	17.5
1997	14.7	17.5	15.7	22.0
1998	14.3	14.4	12.9	18.5
1999	13.6	14.6	13.4	17.6
2000	14.1	13.3	12.4	15.5
2001	14.2	14.4	12.9	18.2
2002	14.3	13.3	12.0	16.9

Table 53. Mortality rate per 100,000 live births according to the reason of death (NSI, 2003)

Year	Prenatal mortality rate per 1000 live births, ‰	Neonatal mortality rate per 1000 live births, ‰	Post-neonatal mortality rate per 1000 live births, ‰	Mortality rate per 100 000 live births according to the reasons:	
				connected with the prenatal period	connected with congenital anomalies
1994	12.0	8.5	7.9	501.0	485.9
1995	11.9	7.8	7.0	464.1	418.2
1996	13.0	8.2	7.4	479.3	458.5
1997	13.3	8.9	8.7	530.2	407.0
1998	13.0	8.2	6.3	d.n.a	d.n.a
1999	12.9	8.3	6.4	d.n.a	d.n.a
2000	12.2	7.5	5.9	438.4	320.3
2001	12.3	7.8	6.7	422.4	327.1
2002	12.6	7.3	6.1	398.5	282.7

Table 54. Mortality rate per 100,000 live births according to reasons connected with the prenatal period, nu (NSI, 1995)

Year/Type of reason	1980	1985	1991	1994
Low birth weight and preterm delivery*	115.5	157.2	162.7	90.6

**Low birth weight: < 2500 g; Preterm delivery: < 37 completed weeks.*

The Sofia Registry of Congenital Anomalies (SORCA) operates according to EUROCAT criteria was organized in 1996 (Simeonov and Dimitrov, 2001). During a four years experiment, 39124 pregnancies have been followed because of fetus/newborn affected of isolated, or multiple, congenital anomalies (CA). In 1.9% of all pregnancies congenital anomalies were established divided between live born – 89.3%, stillborn – 5.1% and induced abortions – 5.6%. Sixty per cent of the registered CA had multifactor and two per cent were with purely environmental etiology. As a conclusion, it was shown that the teratogenic effect is a result of complex interrelationships between environmental and genetic factors.

4.2. Localization of reproductive problems

The bold figures in Table 55 indicate high concentrations of the pollutants and big local emissions. With bold figures concerning infant mortality, rates prevailing over the national averages are stressed. Several regions are detected with higher infant mortality rates than the national average. Some of them are situated in areas with well-developed agriculture or fruit growing (Kjustendil, Razgrad, Sliven). Others are industrialized regions with energy production plants (Plovdiv, Kardjali, Varna, Pernik, Stara Zagora). As the biggest emitters of dioxins, the thermal electrical plants, are situated near Stara Zagora and Kjustendil, whilst the biggest metallurgical plants are near Sofia and Pernik. A village, Galabovo near Stara Zagora, is strongly influenced by the emissions, and is a “hot spot” associated with a lot of infant developmental problems. Kardjali is another “hot spot”. Kardjali and Plovdiv are centers for production of non-ferrous metals. Lead and cadmium, which are known as reproductive toxicants, are emitted by the plants polluting environment

In the period 1997-2001 the mean annual concentrations of cadmium in ambient air of Kardjali exceeded standards by nine to eighteen times, and the standardized concentrations of Cd in the river Arda were exceeded six times (Staykova and Turnovska, 2002). Similarly lead concentrations in the river water exceeded

standardized concentrations by a factor of approximately 2.2 and lead exceeded the ambient air standards in the town by a factor of 1.5. The amount of the metals in the body varied in average between 9 and 40 mg Cd (the last - for smokers) and 10 mg Pb (per 70 kg body weight) (Municipality of Kardgali, 2001). Correlation between the lead concentrations in mothers and babies blood is proved because of transcaul transfer of the metal, especially at professional exposure of the mother, and of a Pb concentration in blood exceeding 400 μg/l. It is known, that the increase of lead concentration in the mothers blood with every 100 μg/l over the initial value of 120-140 μg/l increases the unfavorable consequences connected with reproduction and neuropsychological disturbances of the babies born (Basmadjieva, 1991). The relative risk (RR) of congenital anomalies for Kardjali was calculated to be 4.58 (Petkov, 1996a). Over three years, children are especially vulnerable to the impact of lead. Bad diet, the lack of iron in the food, anemia and bad personal hygiene add additional health risk (Petkov, 1996b).

Table 55. POPs in environment in 2001 and mortality rate in 2002 (MoEW, 2004; NSI, 2003)

Region	DDT_{total}		Emissions of dioxins and furans, g/year	Infant mortality rate per 100000 people due to reasons connected with:		Infant mortality rate per 1000 life-births	
	In ground water, μg/l	In soil μg/kg		Congenital anomalies	prenatal period	Total	In rural areas
Bulgaria	-	-	132.18	3.0	3.4	13.3	16.9
Burgas	n. d.	**954.3**	**8.92**	1.7	1.2	**13.7**	16.4
Varna	n. d.	n. d.	**3.20**	**3.5**	**4.8**	12.6	**20.5**
Vidin	n. d.	n. d.	0.13	1.6	**4.7**	10.1	11.8
Vratca	n. d.	n. d.	0.18	1.4	**3.6**	12.4	**15.8**
Gabrovo	n. d.	n. d.	0.21	**6.3**	1.4	12.9	**33.3**
Dobrich	n. d.	n. d.	0.06	**5.2**	**4.2**	13.2	**19.8**
Kardjali	0,003	**34.5**	0.16	**3.7**	**4.3**	**13.5**	**16.6**
Kjustendil	n. d.	**704.**8	**12.56**	1.9	2.5	**14.6**	9.1
Lovech	n. d.	n. d.	0.22	**3.6**	2.4	**15.5**	14.5
Montana	n. d.	**118.8**	0.04	1.7	**3.9**	12.4	**19.8**
Pazardjik	n. d.	**37.3**	0.11	**3.6**	1.6	12.6	12.5
Pernik	n. d.	**182.8**	**9.84**	2.7	**4.7**	**13.9**	0.0
Pleven	0,012	n. d.	0.10	2.2	1.9	**13.9**	**19.5**
Plovdiv	0,0006	**168.8**	**1.85**	**4.5**	**5.3**	**16.9**	**21.1**
Razgrad	n. d.	**120.0**	0.02	**3.5**	**7.6**	**15.6**	**20.4**
Russe	0,03	**67.2**	0.68	**3.4**	1.1	9.9	7.9

Table 55. continued

Region	DDT_{total}		Emissions of dioxins and furans, g/year	Infant mortality rate per 100000 people due to reasons connected with:		Infant mortality rate per 1000 life-births	
	In ground water, µg/l	In soil µg/kg		Congenital anomalies	prenatal period	Total	In rural areas
Silistra	n. d.	n. d.	0.24	2.1	**3.6**	13.2	16.2
Sliven	0,002	**20.8**	0.86	**6.5**	**9.3**	**32.3**	**38.2**
Smolyan	0,001	**668.1**	0.09	2.2	0.7	5.9	2.4
Sofia	0,005	**252.3**	**17.39**	2.4	2.7	8.9	12.3
Stara Zagora	n. d.	**302.5**	**70.43**	**3.3**	3.3	13.2	**17.4**
Targovishte	n. d.	n. d.	1.22	2.9	7.8	**17.6**	**19.8**
Shumen	n. d.	n. d.	0.20	**4.9**	3.9	**18.1**	**19.8**
Yambol	n. d.	n. d.	0.04	2.6	5.2	**15.7**	**20.2**
Haskovo	0,001	n. d.	**2.27**		1.8	8.4	4.0

n.d. – not detectable

The necessity of urgent measures forced the officers of the lead producing factory in Kardjaly to install the additional cleaning equipment, with filters for the fine removal of the Pb-containing dust from the waste-exhausted gases and the ventilation outlets.

5. Conclusions

Environmental reproductive health in Bulgaria is assessed. Chemical contamination of the environment in Bulgaria is described on the basis of National inventory of selected persistent organic pollutants (DDT and dioxins and furans) and heavy metals (Pb and Cd), which are reproductive toxicants. Comparison of the mean environmental concentrations of DDT in Bulgaria with the standardized concentrations shows that incidentally they exceed standards for ground water, air and soil. The mean annual emissions for unit area of dioxins and furans in Bulgaria are high, and are comparable with countries that are more industrialized. A trend of reduction in emissions is established. Pollution of soil with Pb and Cd is due to old burdens, and is localized in areas with mining and extractive metallurgy, near factories from the ferrous and nonferrous metallurgy, high ways and main roads.

Significant and persistent reduction of the population, as well as a negative population growth is established. Male and female fertility, as well as prenatal and

postnatal developmental problems, are detected. Statistical data for Bulgaria for the period 1994 - 2002 show a slight increase of the stillbirth rates and lack of statistical difference of the spontaneous abortion rates. Male/female ratio among newborns, which is used as an environmental health indicator, shows that from 1920 till now it is almost constant. Trends of reduction in the infant mortality rates are established, but still are higher than the other European countries. On the basis of data from SORCA, Bulgarian authors have concluded that the teratogenic effects were results of complex interrelationships between environmental and genetic factors

The reproductive problems were localized mainly in places with environmental pollution due to heavy industry, agriculture with illegal use of forbidden pesticides and old environmental burdens. But data show as well that the reproductive problems in Bulgaria are a combination of socio-economic, environmental and genetic factors.

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LIST OF ABBREVIATIONS

A-1254	Aroclor 1254
A-1268	Aroclor 1268
AGE	Advanced Glycation End Product
AGE-R2	80K-H phosphoprotein
AGE-R3	Galectin 3
AHR	Aryl Hydrocarbon Receptor
ALK1	Activin Receptor-Like Kinase 1
AOR	Adjusted Odds Ratio
AR	Androgen Receptor
ART	Assisted Reproduction Technology
ATP	Adenosin tri-phosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BBZP	Butylbenzyl Phthalate
BMI	Body Mass Index
BPA	Bisphenol A
BW	Body weight
CA	Chlorine atom
CALUX	Chemical-Activated Luciferase Expression
Carc-PAHs	Carcinogenic PAHs
CASA	Computer Aided Sperm Analysis
CDC	Center for Disease Control and Prevention
CEC	Commission of the European Communities
CFC	Chlorofluorocarbon
CI	Confidence Interval
CML	N-ε-Carboxymethyl Lysine
CRISMAS	Copenhagen Rigshospitalet Image House Sperm Motility Analysis System
CTGF	Connective Tissue Growth Factor
Cyt-4501A1	Cytochrome-4501A1
DBCA	1,2,-Dibromo-3-Chloropropane
DBP	Dibutyl Phthalate
DDE	Dichlorodiphenyl-dichloroethylene
DDT	Dichlorodiphenyl
DEHP	Di-Ethylhexyl Phthalate
DEP	Diethyl Phthalate
DES	Diethylstilbestrol
DFI	DNA Fragmentation Index
DINP	Di-Isononyl Phthalate
DMP	Dimethyl Phthalate

DNA	Deoxyribonucleic Acid
Dnop	Di-N-Octyl Phthalate
DW	Dry Weight
EC	European Commission
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECM	Extracellular Matrix
ED	Endocrine Disrupters
EDC	Endocrine Disrupting Chemical
EE_2	Ethynylestradiol
EHDI	Estimated Human Daily Intake
ER	Estrogen Recptor
ERBA	Estrogen Receptor Binding Assay
ERE	Estrogen Response Element
ESR	Estrogen Receptor
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
EU	European Union
F0	Parent Generation
F1	First Generation of Offspring
F2	Second Generation of Offspring
FISH	Fluorescence *In Situ* Hybridization
FSH	Follicle Stimulating Hormone
GFN	Genitofemoral Nerve
Ghrh	Growth Hormone Releasing Hormone
GSH	Glutathione
HCH	Hexachlorcy clohexane
HMGB1	High-Mobility Group Protein-1
HNRA	Heterogeneous Nuclear RNA
HPLC	High Pressure Liquid Chromatography
HPRT	Hypoxanthine-Guanine Phosphoribosyl-Transferase
HPT	Hypothalamo-Pituitary-Testicular Axis
HSDB	Hazardous Substances Database
HUVEC	Human Umbilical Vein Endothelial Cells
IARC	International Agency for Research on Cancer
ICSI	Intracytoplasmatic Sperm Injection
IFN-gamma	Interferon gamma
IG	Immuno Globulin
Ig G	Immunoglobulin G
IL	Interleukin
IMPY	2-Isopropoxy-4-Methyl-Pyrimidinol
INF	Interferon
INSL3	Insulin Like Growth Factor 3
IPCS	International Programme on Chemical Safety
IQR	Interquartile Range
IRR	Incidence Rate Ratio
IUGR	Intrauterine Growth Retardation

IVF	*In vitro* Fertilization
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LEY I-L	Leydig Insulin-Like Protein
LGR8	Leucine-Rich Repeat-Containing G Protein-Coupled Receptor 8
LH	Luteinizing Hormone
LIN	Linearity
LOAEL	Lowest Observed Effect Level
LOX	Lysyl Oxidase
Lp(a)	Lipoprotein (a)
MBP	Monobutyl Phthalate
MBT	Monobutyltin
MBZP	Monobenzyl Phthalate
MEHP	Mono (2-Ethylhexyl) Phthalate
MEP	Monoethyl Phthalate
MET	Maternal-Embryonic Transition
MFO	Mixed Function Oxidase
MIS	Müllerian Inhibiting Substance
MMP	Monomethyl Phthalate
MRNA	Messenger Ribose Nucleic Acid
MVLN	Breast cancer cell line, stably transfected with an ERE driven luciferase reporter plasmid
NF-kB	Nuclear factor-B
NGOs	Non-Governmental Organization
NHANES	National Health Nutrition and Examination Survey
NOAEL	No Observed Adversed Level
NOx	Nitrogen Oxides
O	Oxygen
OECD	Organisation of Economic Cooperation and Development
OR	Odds Ratio
OSPAR	Convention for the Protection of the Marine Environment of the North-East Atlantic Region
OST-48	Oligosaccharyl Transferase 48
P,P'–DDE	1,1-Dichloro-2,2-Bis(P-Chlorophenyl)Ethyle
PAH	Polycyclic Aromatic Hydrocarbons
PAI-1	Plasminogen Activator Inhibitor-1
PBB	Polybrominated-Biphenyl
PBDE	Polybrominated-Diphenyl Ether
PCB	Polychlorinated Biphenyl
PCDD	Polychlorinated -Dioxin
PCDF	Polychlorinated Dibenzofuran
Pcdf	Polychlorinated-Furan
PCDF	Proper Chemical Disposal Fact Sheet

PCNs	Polychlorinated-Naphtalenes
PCOS	Polycystic Ovary Syndrome
PFOA	Perfluorooctane Octanoate
PFOS	Perfluorooctane Sulfonate
PGC	Primordial Germ Cell
PM_{10}	Particulate Matter <10 μm in aerodynamic diameter
POP	Persistent Organic Pollutant
Pp'dde	Pp' Dichlorodiphenyl-dichloroethylene
PPB	Parts Per Billion
PRL	Prolactin
R49X	Arg49-To-Ter Nonsense Mutation
RAGE	Receptor for AGE
REACh	EU regulatory framework for the Registration, Evaluation and Authorisation of Chemicals
RfD	Reference Dose
RLF	Relaxin-Like Factor
SCF	European Scientific Committee on Food
SCSA	Sperm Chromatin Structure Assay
SD	Standard Deviation
SG	Specific Gravity
SHBG	Sex Hormon Binding Globulin
SO_2	Sulfur Dioxide
T	Testosterone
T_4	Thyroxine
TBT	Tributyltin
TBTO	Tributyltin oxide
TCDD	Tetrachlorodibenzo -p-dioxin
TCPY	3,5,6-Trichloro-2-Pyridinol
TDI	Tolerable Daily Intake
TDM	Tail Distributed Moment
TDS	Testicular Dysgenesis Syndrome
TEF	Toxic Equivalent Factor
TEQ	Toxic Equivalent
TGD	Technical Guidance Document
TGF-beta	Transforming Growth Factor-Beta)
Tgz	Troglitazone
TNF	Tumor Necrosis Factor
TNFalpha	Tumour Necrosis Factor-alpha
TSH	Tyroid Stimulating Hormone
US EPA	United States Environmental Protection Agency
US NTP	United States National Toxicological Program
USA	United States of America
VAP	Average Path Velocity

VCAM-1	Vascular Cell Adhesion Molecule-1	VSL	Straight Line Velocity
VCL	Curvilinear Velocity	WHO	World Health Organisation

LIST OF UNITS

Prefixes to Units

da	deca	(10^{1})	d	deci	(10^{-1})
h	hecto	(10^{2})	c	centi	(10^{-2})
k	kilo	(10^{3})	m	milli	(10^{-3})
M	Mega	(10^{6})	µ	micro	(10^{-6})
G	Giga	(10^{9})	n	nano	(10^{-9})
T	Tera	(10^{12})	p	pico	(10^{-12})
P	Peta	(10^{15})	f	femto	(10^{-15})

Units

°C	degree Celcius or centigrade	pa	per annum
		pH	acidity
d	day	ppb	parts per billion
Drachme	Former Greek currency unit	ppm	parts per million
		s	second
Euro	European currency unit	t	ton
g	gram	te	ton emission gas
h	hour	tpa	ton per annum
kg_{bw}	kilogram body weight	US$	US Dollar
kgpa	kilogram per annum	y	year
l	litre		
m	metre		
Nm^3	Normalised cubic metre		

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