What Should It Take To Describe a Substance or Product as "Sperm-Safe"

David Mortimer[1], Christopher LR Barratt[2], Lars Björndahl[3], Christiaan de Jager[4], Anne M Jequier[5] and Charles H Muller[6]

[1] Oozoa Biomedical Inc, Box 93012 Caulfeild Village, West Vancouver, BC, V7W 3G4, Canada.

[2] Reproductive and Developmental Biology, Medical School, Ninewells Hospital, University of Dundee, Dundee, DD1 9SY, Scotland, UK.

[3] Centre for Andrology and Sexual Medicine, Karolinska University Hospital and Karolinska Institutet, Huddinge, 141 86 Stockholm, Sweden.

[4] School of Health Systems and Public Health, University of Pretoria, Pretoria, South Africa.

[5] PIVET Medical Centre, 166 Cambridge Street, Leederville, Western Australia 6007, Australia.

[6] Male Fertility Laboratory, Department of Urology 356510, University of Washington, Seattle, WA 98195-6510, USA.

Corresponding author: Dr David Mortimer
Oozoa Biomedical Inc, Box 93012 Caulfeild Village, West Vancouver, BC, V7W 3G4, Canada
E-mail: <david@oozoa.com> Tel: +1 604 926 9570 Fax: +1 604 926 9571

Table of Contents

Abstract

BACKGROUND: Male reproductive potential continues to be adversely affected by many environmental, industrial and pharmaceutical toxins. Pre-emptive testing for reproductive toxicological (side-)effects remains limited, or even non-existent. Many products that come into direct contact with spermatozoa lack adequate testing for the absence of adverse effects, and numerous products that are intended for exposure to spermatozoa have only a general assumption of safety based on the absence of evidence of actual harm. Such assumptions can have unfortunate adverse impacts on at-risk individuals (e.g. couples who are trying to conceive), illustrating a clear need for appropriate up-front testing to establish actual "sperm safety".

METHODS: After compiling a list of general areas within the review's scope relevant literature and other information was obtained from the authors' personal professional libraries and archives, and supplemented as necessary using PubMed and Google searches. Review by co-authors identified and eliminated errors of omission or bias.

RESULTS: This review provides an overview of the broad range of substances, materials and products that can affect male fertility, especially through sperm fertilizing ability, along with a discussion of practical methods and bioassays for their evaluation. It is concluded that products can only be claimed to be "sperm-safe" after performing objective, properly designed experimental studies; extrapolation from supposed predicate products or other assumptions cannot be trusted.

CONCLUSIONS: We call for adopting the precautionary principle, especially when exposure to a product might affect not only a couple's fertility potential but also the health of resulting offspring and perhaps future generations.

Key words: spermatozoa; toxicity; safety; male fertility; pharmacology

Introduction

It is well-established that male reproductive potential (commonly "fertility") has been, and continues to be, adversely affected by many environmental, industrial and pharmaceutical toxins (e.g. Perry, 2008; Jurewicz et al., 2009; European Science Foundation, 2010; Joffe, 2010; Sharpe, 2010; Perry et al., 2011; Sutton et al., 2012). Unfortunately, the reproductive toxicity of the great majority of these problem substances was discovered after-the-fact, and pre-emptive testing for reproductive toxicological (side-)effects remains limited, or even non-existent. With the burgeoning markets for nutraceuticals, processed food and food packaging, cosmetics and personal care products, it therefore seems apposite that awareness be created regarding such risks to human and animal fertility. Beyond the many substances and materials that might affect male fertility indirectly, many products that come into direct contact with spermatozoa are reaching market without adequate testing for absence of adverse effects, even some that receive CE marking (*Conformité Européenne*; a mandatory conformity mark for products placed on the market in the European Economic Area) for use in assisted conception treatment. For example, saying that a product is "non-spermicidal" only means that it does not specifically contain a spermicidal drug (Vargas et al., 2011), and there are numerous products that are intended for exposure to spermatozoa where there is nothing documented or known, only a general assumption of "safety" based on the absence of any evidence of actual harm. By providing an overview of the broad range of substances, materials and products that can affect male fertility via sperm production or sperm fertilizing potential, we hope to raise awareness of the extent of the problem (Sutton et al., 2012) and the increasingly urgent need for action to avoid personal calamities by individuals (e.g. couples who are trying to conceive) on the one hand, and severe environmental consequences on the other.

Further information to facilitate understanding of this review by non-spermatologist / non-andrologist readers can be found in basic andrology reference books, including Hargreave (1994a); Mortimer (1994), Patton and Battaglia (2005); Schill et al. (2006), Björndahl et al. (2010), Nieschlag et al. (2010a) and Jequier (2011).

Methods

Given the recognition that many substances and materials that might affect male fertility potential either directly or indirectly are reaching market without adequate prior testing for adverse effects on spermatozoa, an international group of authors with extensive experience in spermatology and andrology was formed with DM acting as the coordinating author. Following an initial series of telephone conversations and e-mail exchanges, the following general objectives were set for the article:

a) To compile a comprehensive, critical overview of the physical and chemical factors that can influence human male fertility by affecting sperm production and/or sperm functional potential.

b) To expand this list to include pharmaceuticals, nutraceuticals, processed food and food packaging materials, cosmetics and personal care products, and products used in assisted conception treatment.

c) To focus primarily on human male fertility, but to augment our understanding using research from other animal species when necessary.

d) To make the article understandable by readers from a broad range of backgrounds, many of whom would not have specialist knowledge of sperm physiology and pathophysiology.

Because of the necessarily broad scope of the article, an initial manuscript outline was created based on a list of sections that represented the general areas of interest within its scope. The outline for each section was then expanded with lists of topics and sub-topics, reviewed and amended by all authors, and then used as the framework for compiling the content. Each author either self-assigned or was asked to contribute to various sections, based on their particular areas of interest, experience and expertise. Relevant literature and other material for each (sub-)topic was obtained from personal professional libraries and archives, and supplemented as necessary using PubMed and Google searches to ensure breadth of coverage. While the bibliography of resources employed is extensive, the vast scope of all the pertinent issues preclude its being exhaustive for simple reasons of practicality. Review by co-authors was considered to have adequately identified and eliminated any errors of omission or bias. Summarization of material was based on the expertise and the contributing author for each (sub-)topic, followed by review and acceptance by all co-authors.

At several points during its preparation, the manuscript was circulated to all co-authors for overall review and comment on both specific content and general readability. The coordinating author compiled all revisions and suggestions using the principle of unanimity based on implicit acceptance in the absence of contrary comment, combined with consensus established via e-mail polling and other exchanges. The final version of the manuscript for submission, as well as the revised version following feedback from the reviewers, including all summaries and recommendations, was approved by all authors.

Factors affecting male reproductive capacity

Male reproductive function is claimed to be decreasing (Carlsen et al., 1992; Sharpe and Skakkebaek, 1993; Sharpe, 2012), specifically a perceived decline in sperm counts in the ejaculates of normal men. However, the extent to which measurements of sperm concentration in complete and incompletely collected semen samples is a reliable measure for the fertility status of entire populations has been hotly debated (Mahmoud et al., 1997; Gottardo and Kliesch, 2011; Isobe, 2012). Based on reported decreases in sperm concentrations in the general population, and decreased sperm motility and morphology in the male partners of couples seeking infertility treatment it has been hypothesized that environmental chemicals with estrogenic properties are negative factors for male fertility (Tas et al., 1996; Van Waeleghem et al., 1996; Phillips and Tanphaichitr, 2008; Diamanti-Kandarakis et al., 2009; Giwercman, 2011; Woodruff, 2011), even though no apparent and clear decrease in population fertility has been noted in epidemiological studies (Akre et al., 1999; Scheike et al., 2008; Joffe, 2010). Nonetheless, although the epidemiological consequences are unclear, there may be toxicological effects on individuals, since both clinical and laboratory research suggests that all the changes in male reproductive health appear inter-related and may have a common origin in fetal life or childhood (Skakkebaek et al., 2001; Buck Louis et al., 2008, Skakkebaek et al., 2011) and several epidemiological studies implicate exposure to environmental chemicals, mainly endocrine disruptors, in male reproductive health disorders (Sharpe, 2009, 2010; Balabanič et al., 2011).

The mechanisms whereby xenogenic factors exert their adverse effects on male reproduction through events occurring during fetal testis and germinal cell development, commonly referred to as the "testicular dysgenesis syndrome", is clearly outside the scope of the present review. However, many effects are mediated either by targeting pituitary gonadotropins (Mutoh et al., 2006) or via the genetic regulation of steroidogenesis (Kuhl et al., 2007) at either the genomic (Liu et al., 2005) or proteomic (Laier et al., 2006; Klinefelter et al., 2012) levels. Gene pathways disrupted included cholesterol transport and steroidogenesis, as well as pathways involved in intracellular lipid and cholesterol homeostasis, insulin signalling, transcriptional regulation, and oxidative stress (Liu et al., 2005). Other gene targets include alpha inhibin (which is essential for normal Sertoli cell development) and genes involved with communication between Sertoli cells and gonocytes (Liu et al., 2005). Environmental toxicants can induce tissue oxidative stress and peroxidation (Kabuto et al., 2004) and induce germ cell apoptosis in the human fetal testis (Coutts et al., 2007).

This concept of a "critical period of exposure" applies to both the gonad and brain, as illustrated by the diethylstilbestrol experience in humans and experiments in rodents showing that estrogenic compounds, present during a critical pre- or peri-natal period can influence behaviour, accessory glands and reproductive structures (Harris and Waring, 2012). Other compounds present at these times can have effects that are not necessarily hormonal in nature, and are manifested later in life, particularly, epigenetic modifications (Anway et al., 2005, 2006).

Emerging trends in the male reproductive health of wildlife also raise the possibility of environmental factors as partial etiologic contributions to the observed reproductive health decline (Edwards et al., 2006; Hamlin and Guillette, 2010). Experimental results and clinical reports have suggested that prenatal exposure to exogenous estrogens may play an etiologic role in the trends observed in male fertility (Danish Environmental Protection Agency, 1995; Toppari et al., 1996; Skakkebaek et al., 2011; Braw-Tal, 2012). While awareness of the biological risks of chemical toxicity has increased considerably in recent years (Zhu and Huang, 2011; Rhomberg and Goodman, 2012) (Table 1), some of these chemicals have long half-lives and have been detected in environmental samples 10–20 years after they were banned for sale or use (Aitken, 2004).

A short list of those substances and physico-chemical factors that have established direct effects on sperm function in vitro, and hence are of particular interest within the context of the present review, has been provided in Table 2.

Environmental endocrine disrupting chemicals

Mankind and wildlife alike are exposed to many general classes of chemicals present in the natural environment, including pesticides, fungicides, heavy metals, defoliants and other chemical weapons, as well as oils and cleaning agents (Colborn et al., 1993, 1997; Sheiner et al., 2003; Gore, 2007; Woodruff et al., 2008; see also http://www.ourstolenfuture.org/basics/chemlist.htm). Many of these chemicals likely exert their effects through mechanisms involving disruption of the endocrine system ("endocrine disruptor chemicals" or EDCs), which have received a great deal of attention since the introduction of the term "endocrine disruptor" nearly two decades ago (Colborn et al., 1993).

Endocrine disruption refers to a mechanism of toxicity that hinders the ability of cells, tissues and organs to communicate hormonally (Silva et al., 2010), resulting in a wide variety of adverse health outcomes including reduced fertility and fecundity (Giwercman, 2011), spontaneous abortion, skewed sex ratios within the offspring of exposed communities (Yiee and Baskin, 2010), male and female reproductive tract abnormalities (Bornman et al., 2010; Newbold, 2011; Dunbar et al., 2012), precocious puberty (Mouritsen et al., 2010; Deng et al., 2012), polycystic ovary syndrome (Teede et al., 2010), neurobehavioural disorders, impaired immune function and a wide variety of cancers (Skakkebaek et al., 2001; Keinan-Boker et al., 2004; McKinley et al., 2008; Ndebele

et al., 2010). Endocrine disruptors represent a wide range of chemical classes and include agonists of the estrogen receptor, androgen receptor antagonists, and aryl hydrocarbon receptor agonists (Beischlag et al., 2008). Some chemicals exhibit more than one mechanism of action (Phillips and Foster, 2008).

The major groups of environmental chemicals with EDC activity includes the organochlorine pesticides, other pesticides, herbicides and fungicides, polychlorinated biphenyls (PCBs), dioxins and furans, alkylphenol polyethoxylates, bisphenol A, phthalates, phytoestrogens and other xenoestrogens (Colborn et al., 1993, 1997) (Table 1). Many of these chemicals persist in the environment. Some are lipophilic and hence sequestered in adipose tissue and secreted in milk, and others may only be present for short periods of time but at critical periods of development.

A number of insecticides and herbicides are known to cause infertility (Perry, 2008; also http://www.arhp.org/publications-and-resources/clinical-proceedings/RHE/Pesticides, accessed 22 October 2012). For example, the nematocide dibromodichloropropane (DBCP) was for many years used to protect the Hawaiian pineapple crop from destruction by a weevil that attacks it roots, but transference of DBCP into the water supply had devastating impact on human fertility by causing azoospermia. Other insecticides such as dimethyl 1-2-dichlorovinyl phosphate (DDVP), and the polychlorinated biphenyls (PCBs), are similarly gonadotoxic.

Exposure to EDCs may occur through environmental routes (air, soil, water, food) or via occupational exposures (Sharpe, 2009; Woodruff, 2011), and individuals may have multiple exposures that in many cases occur chronically and at low doses. EDCs are very diverse in structure and potency (Damstra et al., 2002). Humans are exposed to low levels of multiple EDCs, the effects of which can be additive, and frequently have body burdens comparable to those that cause abnormalities in other vertebrates (Kortenkamp, 2007; Kortenkamp et al., 2007; Bornman et al., 2010). There are also many spermotoxic and gonadotoxic substances beyond EDCs to which men are exposed within their normal environment or in the workplace. Endocrine disruption must be considered in the context of both individuals and populations, although not every individual within a population may be similarly affected, and EDC effects can be permanent or irreversible (Gore, 2007).

There are a number of mechanisms whereby EDCs can modulate endocrine systems and potentially cause adverse effects (Mnif et al,. 2011). The generally accepted paradigm for receptor-mediated responses involves a hormone binding to its receptor at the cell surface, in the cytoplasm, or within the nucleus, followed by a complex series of events within the classical genomic pathway that lead to interaction of receptors with the DNA by binding to hormone response elements in the target gene promoter area (Gruber et al., 2004). Off-target effects as well as cross-talk may occur as many transcription factors modulate transcription in a DNA-binding independent fashion (Beischlag et al., 2008). Gene expression studies aim to elucidate the effects of disruption on a host of genes, attributed to prenatal exposure, in particular genes associated with reproductive tract development, evidenced by rodent models (Welsh et al., 2008; Gore et al., 2011) as well as postulated in humans (Kalfa et al., 2009; Diamanti-Kanarakis et al., 2012).

Heavy metals

All heavy metals are toxic to the testis and readily damage the seminiferous epithelium (Bonde, 2010; Wirth and Mijal, 2010; Marzec-Wróblewska et al., 2012) and cadmium interacts with the zinc-dependent stability of the human sperm chromatin (Casswall et al., 1987). Salts of arsenic, cadmium and mercury, as well as metals such as lead and antimony, are all highly damaging to spermatogenesis, with cadmium salts specifically damaging the Sertoli cells; their accidental ingestion causes infertility (Boscolo et al., 1985; Benoff et al., 2000). The most toxic are cadmium and arsenic salts, which were historically used to protect roof timbers against dry rot and termite invasion (but are now banned in many countries for this purpose). Industrial exposure to lead can reduce the sperm count (Alexander *et al.*, 1996), and men heavily poisoned by lead can also suffer a reduction in thyroid function as well as decreased cortisol production. Mercury also interferes with spermatogenesis and can damage the epididymal ducts. Heavy metals are also present in some welding fluxes, a possible explanation for the known relationship between welding and infertility. Arsenic and antimony can be found in folk medicines, particularly those from the Indian subcontinent and Traditional Chinese Medicine (Lynch and Braithwaite, 2005).

Solvents

Various organic solvents are also known to cause infertility, including glycol ethers (Cherry et al., 2008) which are used in the printing industry and are also found in some paints (e.g. as used on naval vessels). Perchloroethylene, used in the dry cleaning industry, can also cause subfertility, but its effects on sperm morphology and kinematics are subtle, and their impact on fertility remain unclear (Eskenazi et al., 1991).

Pharmaceuticals

Many pharmaceuticals are known to have male reproductive side-effects expressed through deleterious effects on either the production of spermatozoa and/or their function (Drife, 1987). An excellent reference book by Forman et al. (1996) has not been updated, and an up-to-date reference site does not exist – as witnessed by the regular appeals for information on drug side-effects on the Androloglist server (www.andrology.org/?Links:13101201:Androlog_net). Some agents suppress gonadotrophins and thus secondarily cause infertility, others have a toxic effect upon the spermatogenic epithelium, and some have a direct effect on the spermatozoa themselves. Many classes of drugs or individual therapeutic agents beyond those with erectile dysfunction activity affect fertility or sexuality, only sometimes having adverse effects upon sperm production or sperm function as registered side-effects (Forman et al., 1996). These include psychotropic and central nervous system drugs (e.g. antidepressants, anti-epileptics), antihypertensives, cancer chemotherapy, therapeutic hormones, as well as recreational drugs and drugs of abuse such as tobacco, marijuana and cocaine. Therapeutic drugs (as well as physical agents and environmental toxicants) can affect the integrity of sperm chromatin, inducing structural, genetic and/or epigenetic abnormalities (Delbès et al., 2010; Christensen and Marsit, 2011). While the mechanisms that trigger such damage remain largely unresolved (depending on their nature, chemicals can directly target the DNA, induce oxidative stress, or modify epigenetic elements), a given individual's susceptibility depends on their genetic background, lifestyle and exposure to various (other) insults.

Some specific examples of some common pharmaceuticals that affect sperm production and/or sperm function are provided (in alphabetical order based on their general purpose) to illustrate the risks that might be posed by such therapeutic agents and/or their biological mechanisms of action.

Analgesics: Analgesic administration during pregnancy is a common occurrence; however some of these drugs, as evidenced in animal experiments, exhibit anti-androgenic effects (Kristensen et al., 2011). Intrauterine exposure has been linked to abnormal reproductive development as the sensitive reproductive programming window may be influenced by androgen deficiency, resulting in cryptorchidism, hypospadias, and compromised fertility (Welsh et al., 2008). Opioid analgesics, used to treat acute pain, have a direct effect on human sperm motility in vitro, by decreasing sperm motility and at higher concentrations, immotile spermatozoa was observed (Xu et al., 2012).

Anti-depressants, anti-epileptics and other psychotropic drugs: Several anti-epileptic drugs have been reported to adversely affect human sperm morphology and motility (Isojärvi et al., 2004) and lithium, used to treat bipolar affective illness, inhibits human sperm motility (Raoof et al., 1989). Various psychotropic antidepressant drugs and the original beta blocker propranolol have also been shown to be potent inhibitors of sperm motility, at least in vitro – although whether they have similar adverse effects in vivo remains unclear (Hong et al., 1981b, 1982; Levin et al., 1981).

Calcium channel blockers: These drugs disrupt the movement of calcium ions through calcium channels, and have effects on many excitable cells such as cardiac muscle, the smooth muscles of blood vessels, and neurons. Their most widespread clinical application is as anti-hypertensives, but they are also frequently used to control heart rate, prevent cerebral vasospasm, and reduce chest pain due to angina. Because voltage-gated calcium channels are also involved in the regulation of sperm capacitation and hyperactivation, as well as the acrosome reaction, these drugs can also impair sperm fertilizing ability, both *in vivo* and during IVF (Benoff et al., 1994). As a corollary to this, the extreme elevation of intracellular calcium ions can adversely affect sperm vitality, even to the extent that the mechanism might have contraceptive potential (Kumar et al., 2008). Therefore, any chemical, whether a pharmaceutical or naturally-occurring, that affects the calcium homeostasis of spermatozoa is likely to affect sperm function.

Chemotherapeutic agents: Most of the chemotherapeutic agents act as alkylating agents that covalently join different molecules together and thus interfere with cell function and cell division, thereby damaging the spermatogenic epithelium and thus reduce the sperm count, often to azoospermia (Mitchell et al., 2009; Dohle, 2010). As there is no known way to block this effect upon the testis, and it is difficult to give specific information regarding the possible return of spermatozoa to the ejaculate post-treatment (in many patients sperm production is never resumed, yet in others spermatozoa may unexpectedly be found in an ejaculate), pre-treatment sperm storage is essential (Lass et al., 2001; Tomlinson and Pacey, 2003). Others act as anti-folates and thus target rapidly dividing cells, some also inhibit nucleic acid synthesis. The alkylating agent cyclophosphamide, which is probably the most commonly used chemotherapeutic drug, has the direst effect upon sperm production. The damage done to the testis is usually dose dependant, and as cyclophosphamide is frequently used in combination with other anti-mitotics such therapy invariably decreases the sperm count. High dose cyclophosphamide can also interfere with Leydig cell function and hence cause a reduction in testosterone secretion. With the increasing efficiency of these chemotherapeutic agents, the number of patients with this form of infertility is increasing and it is not at all uncommon for such patients to present in infertility clinics.

The suggestion that pre-pubertal boys were less sensitive to the effects of chemotherapy (Schalet, 1981) led to the use of gonadotrophin releasing hormone (GnRH) analogues prior to chemotherapy. While this possible method for protecting the testis from the effects of both chemotherapy and irradiation is still used today (Wang et al., 2010), it remains controversial. GnRH

analogues are also commonly used in the control of prostate cancer, often administered in depot form, with the side-effect of azoospermia. With some men in their 50s now attending infertility clinics, this situation does occasionally occur.

Colchicine: Commonly used as a prophylactic treatment for gout, colchicine can cause a reversible impairment of sperm production in some men (Haimov-Kochman and Ben-Chetri, 1998; Kirchin et al, 1999).

Finasteride: This anti-androgen, a synthetic 5α-reductase inhibitor (an inhibitor of the enzyme that converts testosterone to dihydrotestosterone), is frequently prescribed in the management of male pattern hair loss (e.g. Propecia, Merck, Sharpe & Dohme, and various generic names; see www.propecia.com, www.propeciacanada.org, www.propeciaaustralia.net, www.propeciauk.com), a practice that is becoming increasingly popular with the creation of more clinics that specialize solely in treating this problem. While an early study suggested that finasteride had no deleterious affect on sperm count (Overstreet et al., 1999) this has been refuted in later reports (Glina et al., 2003; Amory et al., 2007; Collodel et al., 2007). It has also been known for some time that even small doses of finasteride can interfere with sexual function (e.g. Uygur et al., 1998; Irwig and Kolukula, 2011), and can give rise to erectile dysfunction and disturbances in ejaculation. Finasteride is also used in the management of prostate cancer, also at doses that will reduce sperm numbers. Unfortunately, the adverse effect of finasteride on sperm count is often disregarded as a possible aetiology of reduced sperm counts in men attending an infertility clinic.

Phosphodiesterase inhibitors: The idea of employing phosphodiesterase inhibitors such as caffeine, theophylline (dimethylxanthine), pentoxifylline and isobutylmethylxanthine (IBMX), as well as dibutyryl-cyclic adenosine monophosphate (db-cAMP), has been around since the 1970s (Matson et al., 1995; Henkel and Schill, 2003; Publicover and Barratt, 2011). These substances certainly show *in vitro* stimulatory effects on motility and kinematics in poorly motile spermatozoa from some men, as well as on various aspects of sperm function related to capacitation and the acrosome reaction, but the effect can also be detrimental. For example, caffeine adversely affects the more motile spermatozoa (Serres et al., 1982), decreases longevity (Traub et al., 1982), and the use of caffeine stimulation of cryopreserved spermatozoa prior to insemination might be deleterious (Vandeweghe et al., 1982). While pentoxifylline in particular has seen extensive clinical use in ART with beneficial outcome in many series of cases of asthenozoospermia, its indiscriminate use has no benefit (Tournaye et al., 1995) and it can decrease sperm longevity (Tournaye et al., 1994a), presumably due to metabolic "burn out" (Centola et al., 1995). Moreover, the inadvertent exposure of oocytes to pentoxifylline is clearly detrimental and must be avoided (Tournaye et al., 1994b). With the advent and widespread use of ICSI, clinical interest in such sperm stimulation therapies (which are, perforce, off-label use of these drugs) has waned somewhat, although pentoxifylline is still sometimes used to try and stimulate non-progressive or "twitching" motility in testicular sperm to facilitate their selection for use in ICSI (Griveau et al., 2006).

Statins: While major adverse side effects of statins (used to treat hypercholesterolemia) on spermatozoa have not been identified, the question has been raised many times on bulletin boards and list servers, along with anecdotal reports of possible cases where statin treatment might have affected sperm fertilizing ability.

More powerful cholesterol sequestering drugs such as ß-cyclodextrin can promote capacitation at low doses, since removal of cholesterol from the sperm membranes is an integral component of the capacitation process (Visconti et al., 1999), and can adversely affect sperm vitality after longer exposure following the induction of high levels of spontaneous, non-physiological, acrosome reactions (Cross, 1999). Consequently, excessive exposure to cyclodextrins, especially methyl-ß-cyclodextrin, could lead to impaired sperm fertilizing ability as a result of premature acrosome reactions, leading to their possible consideration as contraceptive agents (Visconti et al., 1999).

Sulphasalazine is a sulphonamide antibiotic that has been used to treat inflammatory arthritis and some inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, since the 1940s. In 1979 it was realized that it could cause oligozoospermia and even azoospermia, its spermotoxic action being mediated through its metabolic breakdown product sulphapyridine (Levi et al., 1979; Toovey et al., 1981). Fortunately, all its effects, including reduced sperm production, decreased sperm motility, and increased abnormal forms, are reversible (Forman et al., 1996).

Testosterone and anabolic steroids: Being "the male hormone" testosterone is sometimes given mistakenly to try and treat a low sperm count. While exogenous testosterone or testosterone-like agents can improve libido, its negative feedback effect will obviously reduce sperm production, making it an inappropriate therapy for suspected male subfertility. Treatment with the usual doses of testosterone will totally ablate LH secretion (Amory et al., 2006) and cause a major reduction in the levels of intra-testicular testosterone: indeed testosterone forms the basis of the "male pill" (Martin et al., 2000). While testosterone at typical doses usually reduces the sperm count to zero, and hence can never be recommended for a man with subfertility, it can be administered to infertile men in small doses without a reduction in the sperm count provided it is given as an "add back" preparation alongside hCG. This is often used in patients with hypogonadotrophic hypogonadism that can be associated with a delay in pubertal development as is the case in patients with disorders such as Kallman syndrome and similar disorders.

It must also be remembered that the secretion of naturally occurring testosterone normally shows a marked diurnal change

(Plymate et al., 1989; Waite et al., 2009), being high in the early morning and falling as the day proceeds. Thus often among infertile men testosterone can be low simply because it was measured at the wrong time of day: testosterone is best measured between 08:00 and 10:00 hrs in all patients.

A*nabolic steroids* will also ablate LH secretion and cause azoospermia. Although these agents are very useful in the treatment of obesity and in terminally ill patients with clinical AIDS (Strawford et al., 1999), their abuse (typically illicit use) by bodybuilders, weight-lifters and other "power" athletes such as sprinters, results in marked adverse effects upon male reproductive function and serious general health side-effects. Anabolic androgens replace the natural androgens and, like an excess of naturally occurring testosterone, suppress the production of LH via a negative feedback effect, substantially reducing the production of natural testosterone.

Other drugs that raise prolactin and have a similar effect are some of the hypotensive agents as well as the long-term use of chlorpromazine. Less commonly, the drug phenytoin, used in the treatment of epilepsy, also reduces the sperm count probably due to its action in the reduction of LH secretion (Murialdo et al., 1994) although phenytoin is little used nowadays.

Recreational drug use and abuse

Use of illicit drugs is a well-established cause of male infertility, with *marijuana* being one of the most commonly used drugs (Fronczak et al., 2011). Δ-9-tetrahydrocannabinol (THC), the primary psychoactive cannabinoid in marijuana, reduces gonadotrophin secretion, particularly LH (Smith and Asche, 1987), thereby depressing testosterone secretion by the testis (Kolodny et al., 1974) and the sperm count falls as a consequence. THC markedly reduces progressive motility in human spermatozoa, as well as their ability to undergo the acrosome reaction (Whan et al., 2006). In mice THC attenuates sperm motility and male fecundity (Morgan et al., 2012).

Opiates, including *heroin* and *morphine*, are another indirect cause of gonadotrophin reduction. Most legal opiate use is limited in dosage and duration, being confined to some post-operative period or to terminally ill patients with a limited life span. Prolonged use of opiates is usually illicit, e.g. heroin addicts, and their intake is often excessive. Large doses of opiates have an anti-dopamine effect and raise the level of prolactin in serum that in turn reduces LH secretion (Torre and Falorni, 2007) and thus the level of testosterone secretion; a similar effect may be seen in marijuana users. Since the testosterone level can be returned to normal by administration of hCG suggests that opiates do not have any affect on the testis itself.

Cocaine is a central nervous system stimulant and, in moderate doses, suppresses both serum LH and prolactin. There are no major studies of the effects of cocaine on human reproduction, but it has been suggested that abnormal sperm counts are significantly more common among cocaine users than non-users (Bracken et al., 1990).

Alcohol has a mixed action, affecting both spermatozoa and the spermatogenic epithelium of the testis via reducing testosterone synthesis by the Leydig cells (Johnston et al., 1981). A further action that can affect consumers of large amounts of alcohol is damage to the liver. Cirrhosis of the liver results in decreased metabolism of the steroid hormones, particularly oestrogen, resulting in the development of gynecomastia and skin changes known as spider naevi. As the levels of oestrogen rise the gonadotrophin levels fall and the sperm count also falls. There are also direct effects of alcohol as a component of lotions or lubricants on sperm viability.

Smoking cigarettes reduces sperm production and increases oxidative stress, DNA damage and lipid peroxidation levels (Linschooten et al., 2011; Fariello et al, 2012). Spermatozoa from smokers have reduced fertilizing capacity, and embryos display lower implantation rates (Soares and Melo, 2008). Even *in utero* exposure to tobacco constituents leads to reduced sperm count in adult life (Jensen et al., 2004.) Recent male smoking is associated with significantly decreased live birth rates even after adjusting for confounders (Fuentes et al., 2010). In vitro studies using cigarette smoke extract revealed suppression of sperm motility in a concentration- and time-dependent manner as well as an increased number of spermatozoa with low mitochondrial membrane potential (Calogero et al., 2009). In addition, cigarette smoke extract has detrimental effects on sperm chromatin condensation and apoptosis, inducing concentration- and time-dependent increases in the number of spermatozoa with phosphatidylserine externalization (an early apoptotic sign) and fragmented DNA (a late apoptotic sign) (Calogero et al., 2009). Given the adverse effects of cigarette smoking by the male partner on assisted reproductive techniques and the transmission of smoking-induced sperm DNA alterations to pre-implantation embryos, which may predispose offspring to a greater risk of malformations, cancer and genetic diseases, men seeking to become fathers should give up smoking.

Nicotine also has established adverse effects on fertility. It can cause sexual dysfunction due to arteriosclerotic changes in the vessels of the penis, and the consequent development of erectile failure. Nicotine can also worsen infertility due to other causes (the so called "co-factor effect") where smoking may exacerbate the action of a varicocele (Peng et al., 1990).

Nutraceuticals

There are many claims that various nutraceuticals such as acetyl-carnitine, zinc, folic acid, selenium, vitamins C and E, and various antioxidants can improve sperm production and/or quality, primarily sperm motility, endpoints that are all too often used as surrogates for actual fertility (Comhaire and Mahmoud, 2003; Showell et al., 2011). Prospective, randomized, placebo-controlled trials, especially ones of sufficient size for confident interpretation of their results, are rare (Cavallini et al., 2004).

Antioxidant therapy is often proposed, and dietary antioxidants might be beneficial in reducing sperm DNA damage, particularly high levels of DNA fragmentation, although their mechanism of action has not been established and most of the clinical studies are small (Zini and Al-Hathal, 2011; Zini et al., 2009). While *in vitro* antioxidant supplements have been shown to protect sperm DNA from exogenous oxidants, effectiveness in protecting sperm from endogenous ROS, sperm processing and cryopreservation has not been established. A recent Cochrane review concluded that antioxidant supplementation in subfertile males might improve the outcomes of live birth and pregnancy rate for subfertile couples undergoing ART cycles (Showell et al., 2011).

Everyday products that can affect spermatozoa

Beyond the wide range of environmental and workplace substances, pharmaceutical and similar compounds that affect sperm production and sperm physiology in humans and other Eutheria there are many other substances and products to which men are exposed within their everyday lives that can have similar effects, even to the extent of causing subfertility or even sterility.

Food and beverage containers and packaging materials: Much of our exposure to EDCs occurs through what we eat and drink – chemicals such as plasticizers can migrate from food or beverage packaging. Packaging can interact with the packaged food by diffusion/migration processes usually depending on the chemical properties of the food contact material, temperature during treatment and storage, exposure to UV light, and storage time of the product (Muncke, 2009). Even though food packaging contributes significantly to human EDC exposure, the role of food and beverage packaging as an additional source of EDC exposure received little attention until recently.

The use of *bottled water* in the world has doubled in the last 8 years (Ceretti et al., 2010). The majority of mineral water brands (80%) are sold in plastic polyethylene terephthalate (PET) containers. To optimise the properties of packaging material a variety of additives, such as stabilizers, antioxidants, coupling agents and pigments are used in the formulation, e.g. di(2-ethylhexyl)phthalate (DEHP) is a plasticizer widely used in polyvinyl chloride (PVC) products as well as in PET products (ATDSR, 2002). Although PET is a material characterized by elevated chemical inactivity, a number of studies indicate that different storage conditions (sunlight, temperature and duration of each) can contribute to the migration of chemicals from bottles to water (Pinto and Reali, 2009). *Bisphenol-A* is used extensively in many different types of food packaging (Muncke, 2009), and bisphenols and their derivatives with epoxy or chlorohydrin groups are known EDCs in humans and are also potentially carcinogenic (Poças et al., 2007; Vandenberg et al., 2007; Sheng and Zhu, 2011).

It has been reported that around 70% of overall consumer packaging consumption is used for food and beverage packaging. The majority of metal cans have polymeric coatings while paper or carton packaging is often coated or laminated with plastics (Castle, 2007; Muncke, 2009). Plastic food packaging films are used for domestic purposes to wrap foods and also to reheat in a microwave (Inoue et al., 2001). Food is a major exposure route for EDCs. Typical food contaminants include pesticides, dioxins, PCBs, PBDEs, methylmercury, lead and arsenic, which are well characterized in food, with relatively high international public and regulatory awareness (Muncke, 2009).

Phthalates are a group of industrial chemicals with many commercial uses which include paints, personal care products and most commonly as plasticizers in medical devices and food packaging (ATDSR, 2002; Latini et al., 2006). These plasticizers are not covalently bound to the plastic material and are consequently released into the environment with time and use (Latini et al. 2006). Phthalates have substantial adverse effects upon reproductive health (Jobling et al., 2011; Lambrot et al., 2009), including semen quality (Huang et al., 2011). The migration of plasticizers from plastics into food has been studied, in particular some phthalates e.g. di(2-ethylhexyl)adipate (DEHA), which was found in foods wrapped in PVC film (Inoue et al., 2001).

Cling wrap can quickly leach high amounts of p-nonylphenol into vegetable oil and thereby adversely affect the seminiferous tubules, sperm production and epididymides (Bornman et al., 1997; de Jager et al., 1999a). Maternal exposure resulted in impaired general growth and male offspring had reduced testicular mass indicating a direct toxic effect on the testis in animals exposed to p-nonylphenol during fetal life, postnatal period and after weaning (de Jager et al., 1999b).

Personal hygiene and skin care products: Moisturizers contain substances designed to add or retain water, and often also to overcome friction. Typical combinations include small molecular weight alcohols such as glycerol or propylene glycol and/or oils, which make them hyper-osmotic (Richardson and Sadleir, 1967; Critser et al., 1988; Gilmore et al., 1995; Katkov et al., 1998). Many oils and alcohols are toxic to spermatozoa and, like soaps and detergents, permeabilize or dissolve the sperm plasma

membrane (Ozgur et al., 1995; Otsuki et al., 2007; Morbeck et al., 2010). Although spermatozoa are not exposed to these products during normal reproductive activities, exposure can occur when couples are either experiencing difficulty conceiving or are undergoing diagnosis or treatment for subfertility. The major concern is when these products are misused during intercourse by couples who are trying to conceive, and during semen collection for fertility evaluation or treatment (Mortimer, 1994; Björndahl et al., 2010; World Health Organization, 2010).

Reproductive aids: Obviously products intended for contraceptive purposes must be expected to have severe deleterious effects upon spermatozoa, and hence must be avoided by couples who are trying to conceive. However, this is not always clear, and confusion certainly exists in this area.

Spermotoxic products: Contraceptives designed to kill spermatozoa on contact generally contain detergents such as nonoxynol-9 that rupture or dissolve the sperm plasma membrane, leading to an immediate loss of sperm motility and subsequent sperm death. Such compounds are delivered typically either as condom lubricants or contraceptive creams. Nonoxynol-9 (usually used at a concentration of 12.5%) can be present in a number of different forms such as a powder that lines the inside of a condom as well as a solution. At one time such a solution was used to wash out the distal portion of the vas deferens after a vasectomy and thus attempt to reduce the time to azoospermia following this procedure. Unfortunately nonoxynol-9 at higher concentrations produced inflammatory changes in the lower genital tract and the technique was abandoned (Donovan, 1995).

It is not just the chemical toxicity of a substance that is deleterious to sperm survival and function but also the physical properties of the delivery environment. The most effective format for nonoxynol-9 as a contraceptive is as a foam, used either alone as an intravaginal contraceptive or in combination with either a diaphragm or female condom. The foam prevents spermatozoa from reaching the cervix, and the joint actions of the chemical and physical properties of this format gives the combination a very good rating as a contraceptive. Even today when this substance is used both as a foam and in combination with an intravaginal barrier known as the "Sponge"(Pharmatex or Protectaid in USA and Canada, and the "Today Sponge" in the USA), it is as effective as the oral contraceptive which nowadays is considered the 'Gold Standard' in contraception. These nonoxynol-9-containing cream and foams are also marketed under the name of "Delfen II" (Ortho Pharmaceutical Corporation, USA) where the generation of a foam is very much part of the product's contraceptive effect. Any substance that forms a foam in the presence of spermatozoa will be very deleterious to the normal function of those spermatozoa.

Lubricants / intimate moisturizers: This is a particularly interesting category of products that are specifically intended for use by couples who are trying to conceive and are suffering from vaginal dryness: clearly great care must be taken to ensure that their formulations and manufacturing are "sperm safe".

Personal (or vaginal) lubricants are designed to overcome friction, and enhance the comfort of intercourse. Commercial water-soluble lubricants (even ones labelled as "non-spermicidal") typically contain glycerol and/or propylene glycol, with most products having osmolality levels 5–10× the acceptable levels for normal sperm function (Begay et al., 2011; Vargas et al., 2011); elevated concentrations of glycerol have long been known to be spermotoxic (Richardson and Sadleir, 1967; Critser et al., 1988; Gilmore et al., 1995; Katkov et al., 1998Å). Additionally, these products are often "balanced" to the vaginal pH with average pH levels between 4 and 6, and therefore outside the functional range for sperm (Begay et al., 2011; Björndahl *et al.*, 2010; World Health Organization, 2010). Preservatives used in these products can also include calcium chelators (EDTA or citric acid) which can alter sperm function. Even "home remedies" such as baby oil, "mineral oil", and canola oil are toxic to spermatozoa, e.g. by virtue of containing pro-oxidant by-products that can limit sperm function, or from oils interacting with lipophilic membrane constituents causing permeabilization or dissolution of the sperm plasma membrane (Ozgur et al., 1995; Otsuki et al., 2007; Lee and Choe, 2009; Morbeck et al., 2010). It is clear from the literature that all such products should be considered suspect (e.g. Goldenburg and White, 1975; Tulandi and McInnes, 1984; Boyers et al., 1987; Frischman et al., 1992; Miller et al., 1994; Kutteh et al., 1996; Anderson et al., 1998; Agarwal et al., 2008; Vargas et al., 2011).

It must be emphasized that "non-spermicidal" is a drug classification, meaning that a product does not contain a spermicidal drug – it has nothing to do with the sperm-safety of a product. It is of concern that many fertility professionals, including gynaecologists, are unaware of studies showing that "non-spermicidal" lubricants actually harm spermatozoa.

Only the few "intimate moisturizers" specifically intended for use by couples who are trying to conceive – and which have been specifically formulated to be compatible with spermatozoa and sperm function, and proven safe by rigorous validated testing procedures, can be considered as "sperm-safe", e.g. Pre~Seed (INGfertility, Spokane, WA, USA; www.preseed.com).

Ultrasound gels: Vaginal ultrasound examination is commonly used to monitor follicle development in subfertile couples before intercourse or insemination. However, the gels used to "couple" the ultrasound probe with the vaginal wall are known to be spermotoxic (Shimonovitz, et al., 1994; Vargas et al., 2011), and clearly should either be used with great care for transvaginal procedures in fertility patients, or alternative products found and used.

External physico-chemical factors

Although external physical and chemical factors are not "products" that can affect sperm production or come into contact with spermatozoa, their abilities to adversely affect sperm production and/or function make it important that their effects are recognized, especially in regard to designing physiologically appropriate studies and avoiding artefacts.

Temperature and spermatozoa: All textbooks and laboratory guides for semen analysis stress the importance of keeping the ejaculate as close to body temperature as possible during its transport to the laboratory, and the great majority recommend incubating the ejaculate at 37°C to ensure efficient liquefaction (since it is an enzyme driven process) (Mortimer, 1994; Björndahl et al., 2010; World Health Organization, 2010). Exposure of spermatozoa in semen to elevated temperatures greatly reduces their survival, in terms of both motility and vitality, (Makler *et al.*, 1981), exposure to cold, e.g. 4°C, causes a loss of motility but not of vitality (Appel and Evans, 1977; Makler *et al.*, 1981).

There is also the phenomenon of "cold shock", which is due to a combination of irreversible changes in plasma membrane phospholipids and ionic imbalances as a result of reduced enzyme activity, notably calcium loading of the spermatozoa. Lipids can exist in either an ordered "gel" state or a less-ordered (more flexible) "fluid" state, with the transition between these states occurring over particular temperature ranges, which in turn depend on the particular fatty acid composition of the membrane. This latter aspect is why cold shock affects the spermatozoa of some species (e.g. boar) more than others (e.g. bull). The "melting point" of most membranes of mammalian cells is in the range 0–15°C, but is not a "snap" event, resulting in the co-existence of domains of phospholipids in both states, as a consequence of which the membrane undergoes conformational changes that lead to functional changes (White, 1993; Watson, 2000; Sieme et al, 2008; Björndahl et al., 2010).

For the above reasons, semen samples to be used for any type of study must be protected from exposure to temperatures outside the physiological range not just during their transport to the laboratory, but also during their handling, analysis and clinical use.

Temperature and the testis / epididymis: While there are no established adverse effects of cold upon the testis or epididymis until exposure causes frostbite, heating has long been known to have massive impacts on spermatogenesis and spermiogenesis via cryptorchidism. Applying heat to the scrotum reduces both sperm number and quality (Goldstein & Eid, 1989) and applying heat to the testis in experimental animals may even reduce the quality of an embryo at IVF (Mieusset et al., 1992). Although it has been suggested for many years that tight underpants may raise testicular temperature and cause infertility (Brindley, 1982; Zorgniotti, 1982; Zorgniotti et al., 1982), and hence that cooling the testes will restore fertility (Zorgniotti and Sealfon, 1984), there is limited clinical evidence to support such therapy (Zorgniotti et al., 1986). It has also been suggested that varicoceles cause infertility by increasing intra-testicular temperature, and varicocele ligation does appear to lower testicular temperature (Hargreave, 1994b; Nieschlag et al., 2010b; Jequier, 2011). Nevertheless, there is a huge number of men with large varicoceles who have normal semen analyses (Zargooshi, 2007), so heat cannot be the only issue. Recently, concern has been expressed regarding the use of laptop computers, scrotal heating and reduced sperm counts, although at least part of the effect can be attributed to prolonged periods seated with ones legs close together (Sheynkin et al., 2011) a situation analogous to the established problem for long distance drivers (Bujan et al., 2000).

Electromagnetic radiation ("EMR"):

Visible light: Under normal physiological conditions mammalian spermatozoa are not exposed to light in the visible part of the electromagnetic spectrum. Sensitivity of spermatozoa to visible light seems to vary between species, with human spermatozoa not showing any marked adverse effects during exposures of up to 24 h (Mann, 1964; Makler et al., 1980).

Ultraviolet light (UV): Marked adverse effects of UV light on bull, ram and mouse spermatozoa have been known for almost 50 years, based on the intracellular generation of ROS, but human spermatozoa were thought to be resistant to similar doses of irradiation within this part of the spectrum, at least in terms of their motility and vitality (Makler et al., 1980). However, UV (254 nm) irradiation of human spermatozoa caused decreases in sperm motility, progressive motility (including important kinematic measures), sperm vitality and, concomitantly, an increase of the level of lipid peroxidation of the sperm membranes (Torres et al., 2010). Specific information on possible risks to the sperm chromatin as a result of UV irradiation is as yet unavailable.

X-rays: It has long been known that exposure of the testes to even relatively small doses of X-rays results in sterility due to the extreme sensitivity of the seminiferous epithelium to such radiation, causing complete breakdown of spermatogenesis (MacLeod et al., 1964; Mann, 1964). Doses as low as 1–6 Gy can cause reductions in sperm count (Rowley et al., 1974), and 15 Gy (the dose used to treat carcinoma-in-situ of the testis) induces azoospermia. However, irradiation of mature ejaculated spermatozoa has usually little or no effect on their motility, vitality, survival or metabolism but this does not mean that their fertilizing ability, or capacity to generate a competent embryo might not be compromised due to damage to the sperm chromatin (Mann, 1964). Studies on human sperm motility *in vitro* following X-ray irradiation cannot be taken as evidence that X-rays are not harmful to spermatozoa (Makler et al., 1980). Sperm DNA fragmentation as revealed by the Comet assay shows a positive relationship with increasing X-ray exposure over the range 0–2 Gy (Singh et al., 2003).

Radio frequencies: A deleterious effect of high-frequency radio waves on human spermatozoa *in vitro* was first described more than 30 years ago (Makler et al., 1980) but has been of increasing concern over the past few years since it was confirmed that EMR emitted by mobile cellular phones (Agarwal et al., 2008), and more recently wi-fi network signals (Avendaño et al., 2012), can affect semen analysis characteristics. A wide body of evidence now exists showing negative effects of exposure to EMR within this part of the spectrum on human health (Makker et al., 2009). The pathophysiological basis for the adverse effects on spermatozoa has been elucidated as being EMR-induced increased mitochondrial ROS generation causing decreases in both sperm motility and vitality, while stimulating DNA base adduct formation leading, ultimately, to DNA fragmentation (De Iuliis et al., 2009). Notwithstanding the published evidence, the actual extent to which real world cellular phone usage impacts the health or fertility potential of any given man remains elusive – but it certainly remains a cause for concern.

Whole body scanners: Magnetic resonance imaging (MRI) scanners have been considered not to affect spermatogenesis, based on data from mouse (Withers et al., 1985). There has also been no concern regarding risk to the testes or spermatozoa from computerized axial tomography (CAT or CT scans). However, concerns have been raised regarding the new "whole body scanners" now being used in airport security screening, which employ whole body X-ray back scatter imaging. While they operate at relatively low beam energies (28 keV), the majority of the energy is delivered to the skin and the underlying tissue, and hence because of the proximity of the testes to the skin there is considered to be a risk of sperm mutagenesis (Sedat et al., 2010).

Osmolarity: Although the osmolarity of prostatic fluid is generally very similar to blood serum, i.e. between 280 and 300 mOsm, the osmolarity of human seminal plasma is generally accepted to be higher than normal body fluids, and increases with time after ejaculation due to liquefaction, so that by 30–60 minutes at 37°C the typical range is 340–380 mOsm (Velázquez et al., 1977; Gopalkrishnan et al., 1989; Rossato et al., 2002). Exposure to values above 400 mOsm decreases normal sperm function, with a complete cessation of motility >600 mOsm (Rossato et al., 2002). Conditions of 600 mOsm have also been reported to cause reorganization of the actin cytoskeleton in primate spermatozoa inducing sub-lethal flagellar defects (Correa et al., 2007). As noted already, many commercial vaginal lubricants exceed 400 mOsm.

While spermatozoa are apparently readily able to adapt to mild hypertonic conditions (e.g. as in liquefied semen), exposure to hypotonic conditions causes them to take up water and swell, with only sperm showing good metabolic function being able to osmoregulate and survive the swelling (Jeyendran et al., 1984). This osmoregulatory function depends on aquaporins in the sperm plasma membrane, although the exact mechanisms involved remains the subject of current research (Yeung et al., 2010). More extreme stress, or poor osmoregulatory ability results in swelling until the sperm plasma membrane ruptures, killing the cell. It is clearly therefore essential that spermatozoa must be maintained within the usual physiological range of osmotic pressure if their function is to be maintained and protected.

Acidity / alkalinity (pH): Human seminal plasma has a considerable buffering capacity and the pH of human semen is generally accepted as being within the range of 7.2–8.2, although semen pH does tend to increase with time after ejaculation (at least within periods consistent with non-deleterious exposure of spermatozoa to seminal plasma), so that semen pH values of 8.4 are not uncommon. Inflammatory disorders of the prostate or seminal vesicles can result in pH values outside this range. On the whole, human spermatozoa in seminal plasma tolerate elevated (alkaline) pH very well, but are highly sensitive to acidic pH and are killed rapidly when the pH is below 6.8, such as can be experienced within the vaginal milieu following intercourse or pericervical artificial insemination (Mortimer, 1994).

Washed human spermatozoa in culture media are also killed rapidly by acidic pH below 6.8. However, when incubated under capacitating conditions, more spermatozoa undergo acrosome reactions when exposed to sulphate ions at pH 6.8 compared to the generally accepted physiological pH range of 7.2–7.4, and elevated levels of acrosome reactions are also induced at pH $\geq$8.0 (Mortimer, 1995a). Consequently, it is vital that when spermatozoa are being incubated under capacitating conditions, all necessary steps are taken to prevent the pH of the medium exceeding the physiological range of 7.2–7.4. The pH can rise above 7.4 very quickly when a bicarbonate-buffered medium is exposed to room air for more than a few minutes, although the exact critical time will depend on the geometry of the specimen and its interface with the air, which will control the rate of loss of the dissolved CO_2 from the medium (Swain, 2012). For this reason, density gradients to be used for separating spermatozoa from seminal plasma must be prepared in zwitterion-buffered culture media, usually buffered with HEPES, sometimes MOPS (Mortimer 2000; Björndahl et al., 2010).

Culture media for assisted conception procedures: Products intended for use in assisted reproductive technology (ART) applications such as sperm washing for intra-uterine insemination (IUI), *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) are typically tested using *in vitro* assays of sperm motility and survival, but often not for specific sperm functional potential in terms of fertilizing ability. Two scenarios of concern exist: (a) the use of culture media that were not developed or intended for use in therapeutic procedures where sperm function has to be at least maintained, if not promoted; and (b) media intended for ART applications, but which are sub-optimal in their formulations in respect of supporting sperm function.

Sub-optimal ART media formulations: The great majority of human ART media have been, and continue to be, developed based

on efforts to optimize the *in vitro* development of *in vivo* generated mouse zygotes (Mortimer, 2013). While such work typically gives benefit for human embryo development *in vitro*, there have been several occasions where new products intended for the IVF step have had to be withdrawn / reformulated. Many culture media formulated for somatic cells have been used in human ART, although – in retrospect, and compared to more modern formulations – with not necessarily optimum outcomes. For example, Edwards failed to achieve human fertilization *in vitro* until he used Bavister's modified Tyrode's medium (Edwards and Steptoe, 1980; Edwards *et al.*, 1969). More recently, Earle's balanced salt solution, a medium that has been used in human IVF since the earliest days, was demonstrated to be significantly less effective at capacitating human spermatozoa than a medium based on the composition of human oviduct fluid intended for human IVF (Moseley *et al.*, 2005).

Sperm capacitation requires the presence of serum albumin (usually human serum albumin, HSA) as a sterol acceptor, and low HSA levels can impair capacitation, especially in subfertile men. *In vivo*, the protein content of oviductal fluid around the time of ovulation / fertilization is 30 mg/ml, and such a concentration provides excellent support for sperm function studies *in vitro* in a medium formulated to reflect oviduct fluid composition (Mortimer, 1986). Lower albumin concentrations decrease sperm capacitation in vitro and hence diminish the ability of spermatozoa to undergo the acrosome reaction (Mortimer et al., 1989). Reducing the HSA concentration to 10 mg/ml provides adequate support for sperm capacitation in most men within current IVF lab protocols, but 5 mg/ml can lead to an increased prevalence of both low fertilization (<25% inseminated oocytes being fertilized) and failed fertilization at IVF (Mortimer, 2013).

Unphysiological ionic constituents or balances: Spermatozoa can be exposed to these conditions when a non-physiological solution or medium is used to resuspend washed spermatozoa, either following density gradient centrifugation or swim-up migration. Unphysiological ionic balances, especially due to missing essential ions such as calcium or bicarbonate, and/or a metabolic substrate such as glucose, can have rapid, marked adverse impacts on sperm physiology that will result in deleterious effects on not just *in vitro* studies of sperm metabolism, motility, vitality or the component processes of fertilizing ability, such as capacitation or the acrosome reaction, but also their subsequent functional potential if employed in ART treatments (Mortimer, 2013). For such reasons, solutions such as "normal" saline or phosphate buffered saline (PBS) are not appropriate for any clinical application or studies on human sperm physiology or functional competence *in vitro*. Similarly, media developed for somatic cells that include substances that can cause harm to spermatozoa, e.g. through promoting ROS generation (e.g. Ham's F10 medium which contains iron, see Gomez and Aitken, 1996).

Reactive oxygen species (ROS): It has been known for 3 decades that Eutherian spermatozoa generate ROS, primarily in the mitochondria as a result of the monovalent reduction of molecular oxygen during oxidative phosphorylation (Alvarez and Storey, 1982; Holland et al., 1982). Because the sperm plasma membrane has a relatively high content of polyunsaturated fatty acids (PUFAs), they are particularly susceptible to lipid peroxidation by ROS (Alvarez and Storey, 1995). Docosahexanoic acid is one of the main PUFAs, and plays an important role in regulating sperm membrane fluidity; it is also the main substrate for lipid peroxidation (Alvarez and Storey, 1995). Oxidation of docosahexanoic acid is the major factor that determines the motile lifespan of spermatozoa *in vitro* (Aitken and Clarkson, 1987), as well as membrane damage and DNA oxidation (Fraga et al., 1991, 1996).

In semen, most ROS are produced by immature spermatozoa and leukocytes, with the ROS generation capacity of activated leukocytes being several orders of magnitude greater than immature spermatozoa. However, immature spermatozoa may produce comparable levels of ROS in the presence of pro-inflammatory factors (Saleh et al., 2002). *In vivo*, epithelial cells of the epididymis can also generate ROS (Drevet, 2006). The cumulative effect of ROS derived from all sources is negatively correlated with normal sperm function, and directly correlated with male subfertility (Aitken et al., 1998; Agarwal et al., 2008). ROS in human semen can be assayed quantitatively using luminol as a substrate (Björndahl et al., 2010).

The crucial role of ROS in the aetiology of human subfertility is a major area of research, with major reviews being published regularly (e.g. Agarwal et al., 2008; Aitken and De Iuliis, 2010; Aitken et al., 2010; Henkel, 2011). Antioxidants can be efficacious both systemically (Kefer et al., 2009; Gharagozloo and Aitken, 2011; Ross et al., 2010; Showell et al., 2011), and *in vitro* when added to culture media (Chi et al., 2008; Donnelly et al., 2000) and sperm cryopreservation media (Gadea, 2011; Taylor et al., 2009).

Endotoxin: Endotoxins are lipopolysaccharide (LPS) or lipooligosaccharide molecules found in the outer membrane of various Gram-negative bacteria and are an important component of the ability of these bacteria to cause disease. LPS consists of a polysaccharide chain (which is highly variable between different bacterial species) and a lipid moiety, lipid A, which is mainly responsible for the toxic effects, although sperm death can also be caused by a caspase-mediated apoptotic-like effect (Hakimi et al., 2006). Exposure of spermatozoa to LPS induces excessive production of ROS, which have downstream adverse effects on sperm motility (Urata et al., 2001). *In vivo* this is a sequela of genitourinary infection (Sikka et al., 2001), but can also occur *in vitro* when culture media or other products to which spermatozoa are exposed contain endotoxins. Sterile manufacture, or subsequent sterilization of a product does not preclude endotoxin contamination. For obvious reasons, ensuring low endotoxin contamination is a fundamental, essential requirement for all products intended either for use in couples who are trying to conceive or for the manipulation of spermatozoa during ART procedures.

Manufacturing and storage conditions: Products that are designed to come into direct contact with spermatozoa might be "safe" when used in a research setting but might be modified or contaminated during large-scale manufacturing. A bovine sperm motility assay has been used to screen biomaterials (Petzoldt et al., 1985). Plastics, used in production equipment and packaging, are especially prone to release of potentially spermotoxic compounds upon heating, wear and light exposure. Polyvinyl chloride (PVC) is among the most notorious of plastics in this respect since it can leach phthalates, and is used extensively in "medical grade" tubing. Polycarbonate is used in drinking bottles and other uses, and leaches bisphenol A (BPA). Therefore, the design and components of equipment used for production and packaging of sperm-safe products must be carefully chosen and tested to avoid inadvertent spermotoxic contamination of the final product.

Regulatory aspects of reproductive toxicology

Approval processes

The standard approach to regulating toxic compounds such as EDCs is based on hazard assessment and risk assessment. Toxicology testing protocols currently used by regulatory authorities are able to detect endocrine toxicity, and modes of action such as oestrogenic or anti-androgenic are then confirmed using specific endocrine toxicology tests (Marx-Stoelting et al., 2011).

Hazard assessments to support the registration and safe human use of a particular type of chemical begins with necessary mammalian toxicology testing as per international consensus (Bars et al., 2012). These studies usually include: acute single high-dose exposures; chronic toxicity studies in rodents and non-rodents based on repeated dosing for up to 12 months; tests for carcinogenicity in two species of rodent; multi-generation reproductive toxicology; teratogenicity studies in two species; *in vitro* and *in vivo* genotoxicity studies; and further studies as might be required for each particular substance, e.g. testing for neurotoxicity, or evaluation of endocrine effects such as oestrogenicity (Kortenkamp et al., 2012).

Regulatory bodies create specific guidelines that define the data required to support the registration of a chemical. The United States Environmental Protection Agency (US EPA) Office of Chemical Safety and Pollution Prevention (OCSPP), previously the Office of Prevention, Pesticides and Toxic Substances (OPPTS), regulates agrochemicals, public health pesticides and large scale industrial chemicals. The OCSPP provides harmonized guidelines and other documents that specify EPA-recommended protocols to ensure that data meet the required quality standard (see http://www.epa.gov/ocspp/pubs/frs/home/guidelin.htm). Other international regulatory agencies such as the Organization for Economic Cooperation and Development (OECD) have produced similar guidelines and standardized toxicology testing protocols that specify the benchmark for designing acceptable toxicity testing data (see www.oecd.org on "reproductive toxicity"). Protocols such as these include specific recommendations on selecting dosage levels (usually expressed as mg/kg body weight/day), test species, number and gender composition of treatment groups, exposure route and frequency, *in vivo* assessments (clinical observations, body weight, food consumption), haematology and blood chemistry, urine analysis, macroscopic anatomical findings and microscopic histopathology at necropsy. Repeat dose toxicity studies, ranging from sub-acute to chronic exposures, can reveal endocrine toxicity through organ weights, anatomy and histopathology, although standard protocols do not include hormone evaluations.

The route of exposure employed in regulatory toxicity tests must be relevant to how humans might be expected to be exposed. For chemicals the majority of tests employ the oral route, with further testing following dermal or inhalational exposure as appropriate (BfR, 2011). Some extrapolation between routes of exposure in terms of general systemic toxicity is possible, but significant human exposure to a chemical by a route other than oral necessitates specific testing using that route (Bars et al., 2012). Because toxicity depends on total dose over time, valid regulatory testing requires exposure to the maximum tolerated dose (MTD), making appropriate selection of dosing levels an important factor in study design (Harvey and Everett, 2006).

Regulatory endocrine toxicity tests

Based on the US Food Quality Protection Act, which specifically identified the risk that EDCs pose to human health, the US EPA has introduced a programme of additional endocrine toxicity testing. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) provides recommendations on identifying the endocrine disrupting effects of chemicals (study designs, endpoints, validation and data interpretation: see http://epa.gov/endo/pubs/edspoverview/edstac.htm), and various protocols are now undergoing validation. The EDSTAC recommended two tier testing process has been designed to combine both environmental effects and mammalian testing that can be extrapolated to human safety. It will also provide a framework for expanding toxicology databases to include the many thousands of chemicals that come under the US EPA's regulatory remit (Harvey and Everett, 2006).

Risk assessment

Assessment of a new chemical entity should involve a complete toxicology package comprising acute, sub-acute, sub-chronic and chronic toxicity studies in rodents and other mammals, together with carcinogenicity, repro- and genotoxicity studies (ECETOC, 2009). Data are submitted to a regulatory agency (e.g. US EPA) for evaluation and determination of risk assessments that include: critical toxicity; the ***no-observed-adverse-effect-leve***l (NOAEL: *"the greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure"*); and the ***lowest-observed-effect level*** (LOEL: *"the lowest concentration or amount of a substance, found by experiment or observation, that causes any alteration in morphology, functional capacity, growth, development, or life span of target organisms distinguishable from normal /control organisms of the same species and strain under the same defined conditions of exposure"*) in each test species (Marx-Stoelting et al., 2011; Bars et al., 2012; Kortenkamp et al., 2012).

The NOAEL dose (in mg/kg/day) can be extrapolated to define the acceptable daily intake (ADI) for human exposure. For non-pharmaceuticals a 100-fold safety factor is employed by convention, based on two 10-fold factors, one for the extrapolation from animal data to humans, and another to compensate for variability in human sensitivity. Maximum residue limits are set independently, monitored in all representative foodstuffs, and total exposure to residues in food should not exceed the ADI. For agrochemicals, the WHO and the United Nations Food and Agriculture Organization operate the Joint Meeting on Pesticide Residues (WHO-FAO JMPR) to assess toxicology data and confirm ADI levels. This is very useful worldwide since many countries lack comparable regulatory resources (Harvey and Everett, 2006).

Device or drug?

One interesting aspect when considering products that are intended for human exposure is whether they should considered (and hence dealt with from a regulatory perspective) as devices or drugs. While pharmaceuticals are considered to achieve their principal action by pharmacological, metabolic or immunological means, medical devices act by other modes of action such as physical, mechanical, physico-chemical or chemical means.

There is no single, precise definition, of a "drug" as different meanings have been created within law (e.g. drug control law), government regulations, medicine, and even colloquial usage. In pharmacology, a drug is "a chemical substance used in the treatment, cure, prevention, or diagnosis of disease or used to otherwise enhance physical or mental well-being" (http://dictionary.reference.com, accessed 22 October 2012). Moreover, a drug can be a natural bodily product that has been extracted with or without purification, or manufactured externally and then administered for medical purposes, e.g. insulin. In regulatory considerations, a substance is typically considered to be a "drug" if it alters normal bodily – or, by extension, cellular – function. One substance of particular interest in this regard for sperm physiology is hyaluronate, a.k.a. hyaluronic acid or hyaluronan, which does interact with spermatozoa at the cellular level.

Of particular concern in the present context is when a drug is used "off-label", i.e. for a purpose or indication (or at a dose) other than for which it has received regulatory approval – and hence often in an area for which, in all likelihood, no safety or side-effect data have been established through controlled clinical trials.

How are substances identified as hazardous?

Based on historical experience of adverse effects upon human reproductive health, a case could be made from the public health perspective that any substance released (either intentionally or unintentionally) into the environment, encountered in the workplace or home, used as a food additive or as part of a personal hygiene or cosmetic product should be tested for possible gonadotoxic, mutagenic, teratogenic, or spermotoxic actions. However, it has not been possible for every known substance to be tested in advance. For the future, the financial burden of such testing must be weighed against the possible cost of clean-ups, reparations and even punitive damages should a substance affect either human (reproductive) health at the population level, or the fertility of individual couples (e.g. via class action suits).
Cost-effective comprehensive screening methods will be required to protect future human reproductive health and fertility, and while such protocols largely exist for gonadotoxicity, mutagenicity and teratogenicity, such is not the case for evaluating possible spermotoxicity, especially in the rapidly growing markets for personal hygiene or cosmetic products (MarketResearch.com, 2011).

Lists of constituents

There are thousands of chemicals with known EDC and/or toxic effects. When considering a product, a complete list of its components must be known and, for any component known to have spermotoxic activity, its concentration must also be known. Clearly this means all constituents, not just the perceived "active" component(s), need to be known – and also their metabolites and breakdown products, since these could be more harmful than the actual constituent chemical. Several issues can confound

the identification or understanding of potentially dangerous constituents:

Nomenclature: To avoid confusion, both inadvertent and intentional, arising from the multiple names for chemicals, agreement on the use of specific chemical nomenclature rather than "trivial" names is needed. One eloquent example of how different chemical nomenclature systems actually confound understanding is phenoxyethanol, a constituent of some "personal moisturizer" products. But the same compound is also known as "spermicide 741" (see: www.chemindustry.com/chemicals/0599140.html, accessed 8 Jun 2011) and ethylene glycol monomethyl ether (EGME, see www.caslab.com/2-Phenoxyethanol_Ethylene_glycol_monophenyl_ether_CAS_122-99-6/, accessed 8 Jun 2011), when it is readily recognized as having significant toxicity to humans, including suspicions of reduced sperm counts, and affecting female offspring in future generations (Dart et al., 2003; Cherry et al., 2008; Weng et al., 2010).

Same chemical, different purpose: Confusion can also arise when a chemical is used for different purposes in different products. In one situation the chemical might have a specific property that requires its use, while in another situation it can be deleterious, a good example being glycerol. While glycerol is a vital component of sperm freezing media, being an excellent permeating cryoprotectant agent for spermatozoa, it is also known to have deleterious effects upon sperm membranes and also on sperm survival and longevity during prolonged exposure under physiological conditions, both *in vivo* and *in vitro* (Richardson and Sadleir, 1967; Critser et al., 1988; Gilmore et al., 1995; Katkov et al., 1998). Hence glycerol really should not be included in creams or lubricants to which spermatozoa are exposed, especially if they might be used by couples who are trying to conceive.

"Generally regarded as safe": In the absence of specific information as to a chemical's safety for use with spermatozoa, all too often assumptions of presumed safety are made on the basis of whether a chemical is "generally regarded as safe" ("GRAS"), e.g. presuming glycerol to be a safe component of a vaginal lubricant on the grounds that (a) it is a commonly used in skin creams and other lotions, and (b) it is used in sperm freezing media. This *non sequitur* has already been discussed, above.

Testing on both prototypes and production products: There is a possibility that, during product development, a chemical is found not to have a significant adverse effect upon sperm function, but when the final production version of the product is formulated the same chemical might now be a cause of sperm dysfunction. This could arise from a change in the final formulation, where by another component has been added that now allows the expression of an adverse effect, or perhaps a change in the source or type of the original chemical so that it is now of a different purity, or has other minor constituents or contaminants. An example of the latter, a highly purified form of hyaluronic acid might be used in research that leads to the development of a product, but when a commercial version goes into production a cheaper form is used, many of which contain quite high levels of other glycosaminoglycans such as chondroitin sulphates, which can induce artefactual acrosome reactions (Mortimer, 1995a).

Break-down products: These could be the result of chemical modifications to one or more constituents due to improper storage in terms of mishandling, exposure to air or heat, or the age of product. While a manufacturer should not be held responsible for adverse effects that might arise under such circumstances, appropriate warnings should be stated. When developing a product an optimum formulation should take such eventualities into account, and endeavour to minimize such possibilities.

Assessing male reproductive capacity

Toxicants can affect the male reproductive system at a single site or at multiple sites, including the testes, the accessory sex glands, and the central nervous system, including the neuroendocrine system (Table 1). There is no single all-encompassing marker of reproductive capacity in men. Three methodologies with the greatest promise are experimental toxicology, epidemiology, and genetic toxicity (Moline et al., 2000), although integrating information from all three will lead to the broadest understanding of impacts on human health (Woodruff, 2011).

Experimental toxicology

Animal toxicity studies conducted for regulatory submission typically are conducted in rats, mice, rabbits, and dogs with greater focus on rats; observations can be extended across a wide range of exposures in animals, using any route of exposure and any specified dose versus time scenario (Moline et al., 2000). Testing guidelines generally require that common laboratory strains be used and employ at least three dose groups and a control group usually. For most toxicity tests the US EPA requires that the highest dose elicit signs of toxicity without compromising survival.

Reproductive and developmental toxicity testing includes a broader category of endpoints than other kinds of toxicity testing because of the multiple stages of exposure and the variability of possible effects. Exposure of sexually mature animals can cause sterility or decreased fertility by depleting or affecting oocytes or spermatozoa, or by affecting endocrine functions of reproductive organs. If fertilization occurs, abnormalities in the gametes can lead to embryonic death, implantation failure, congenital malformations, embryonic growth retardation, genetic disease, or cancer in the offspring (National Research Council, 2006).

A rodent model is most commonly used for the study of reproductive and developmental toxicity (Wyrobek et al., 1983a; Claudio et al., 1999). For toxicology data derived from animal studies to be used advantageously in risk assessment it is critical to identify and understand species-specific differences in physiology and metabolism that might affect the response to the particular toxicant. The genetic homogeneity of laboratory rodents is advantageous by excluding potential confounding factors, but it can confound the study of susceptible sub-populations unless different strains are studied. Nevertheless, studies in rodents are valuable for hazard identification and determining dose response and critical thresholds for fertility, and are often useful surrogates for human studies, such as in investigating relationships between sperm-based endpoints and function/fertility (Chapin et al., 1997).

The mouse sperm morphology test is highly sensitive to germ-cell mutagens and has potential use for identifying chemicals that induce spermatogenic dysfunction, and also perhaps heritable mutations. A comparison of 25 test chemicals on sperm counts, motility, and morphology in at least two species (including man, mouse and nine other mammals) demonstrated good agreement in response among species (Wyrobek et al., 1983a).

Notwithstanding the development of new specific test guidelines for endocrine effects, the existing repertoire of OECD test guidelines will identify likely endocrine effects pertinent to human health with reasonable certainty through:

- multiple dose testing on endocrine-related tissues ranging from subacute to chronic exposure (e.g. OECD test guideline 407, 409, 451 and 452);
- prenatal toxicity testing in rat or rabbit (OECD test guideline 414) that can reveal disturbances in the development of reproductive organs;
- OECD-SIDS screening tests (e.g. OECD test guideline 421 and 422) investigating endocrine-related tissues specifically designed to detect reproductive effects after relatively short exposure times; and
- one- and two-generation tests (OECD test guideline 415 and 416) which – especially the latter – constitute the most comprehensive investigation of endocrine related tissues and reproductive function (Gelbke et al., 2004).

Detecting a treatment that causes a statistically significant alteration in sperm production, depends on several factors, including the size of the treatment response, the inherent variability in the endpoint of interest among normal, untreated males, and the number of replicate animals per treatment group (Berndtson, 2008).

After determining that a substance is toxic to the male reproductive system further investigation must examine its mechanism(s) of toxicity. Mechanistic information permits predictions regarding the potential toxicity of individual compounds or complex mixtures in humans, better understanding of the windows of vulnerability in the development of the male reproductive system, and for the development of possible preventive or curative measures.

Acute short-term exposure models combined with serial exposure models give a complete picture of the range of effects (Claudio et al., 1999). Exposing animals over a long period of time allows for the detection of transgenerational effects from chemicals, such as male-mediated developmental effects. Multi-generation studies, in particular continuous breeding studies, yield the most thorough assessment of the many complex processes that result in reproductive and developmental toxicity (Moline et al., 2000). Exposure at a critical window of embryonic development can also establish a transgenerational epigenetic effect from a single exposure, as has been shown for the fungicide vinclosolin (Anyway et al., 2005, 2006), Dioxin (Mannikam et al., 2012a), and a mixture of the pyrethroid pesticide Permethrin and the common insect repellent DEET (Manikkam et al. 2012b).

Depending on the nature and purpose of a particular product, other tests of biocompatibility might be also be necessary for it to be considered safe for its intended use. For example, it is not only spermatozoa that can be affected by a vaginal lubricant, but also the vagina, the penis, and even non-genital skin. Consequently, evidence of all appropriate aspects of biocompatibility must be obtained, using established validated methods such as the rabbit vaginal and penile irritation assays, in accordance with relevant regulatory authorities' requirements (International Organization for Standardization, 2010). For example, a mucosal irritation test using slugs has been shown to be more sensitive than the rabbit vaginal irritation model in predicting the vaginal toxicity caused by compounds such as nonoxynol-9 (Dhondt et al., 2004).

Epidemiologic approaches

Epidemiologic methods for assessing the impact of hazardous substances on male reproductive health include questionnaires to determine reproductive history and sexual function, reproductive hormone profiles, and semen analysis (Buck et al., 2004; Tingen et al., 2004). Selection of appropriate methodologies for studying reproductive toxicants requires knowledge of key factors including the nature of the exposed population, the source, levels, and known routes of exposure, the organ systems in which a toxicant exerts its actions, the hypothesized mechanisms of a toxicant's actions, and the techniques available to assess the toxicity in the relevant organ systems (Wyrobek et al., 1997).

To establish the extent of testicular toxicity, researchers can measure the size of the testes, analyze semen samples, or take testicular biopsies, but basic semen analysis – subject to careful standardization and quality control – has been the primary

research tool for studying the effects of toxicants on the male reproductive system (Wyrobek et al., 1983b; Cohn et al., 2002; Brazil et al., 2004a,b). Semen quality has been used as a surrogate for fertility in epidemiologic studies (Slama et al., 2004; Olshan et al., 2007; Jurewicz et al., 2009; Merzenich et al., 2010). Rather than longitudinal studies that require multiple semen samples, cross-sectional epidemiologic investigations using a single semen sample from each participant have been shown to be sufficient if obtained under defined conditions and according to a set protocol (Schrader et al., 1991; Stokes-Riner et al., 2007).

A toxicant or its metabolite may act directly on accessory sex glands, altering the quantity or quality of their secretions, or it can enter the seminal plasma and affect the spermatozoa, or it can be carried to the site of fertilization by the spermatozoa and affect the oocyte or embryo. The presence of toxicants or their metabolites in seminal plasma can be analyzed using atomic absorption spectrophotometry or gas chromatography/mass spectrometry (Moline et al., 2000).

To establish the extent of endocrine dysfunction, hormone levels can be measured in blood and urine. The NIOSH-recommended profile for evaluating endocrine dysfunction associated with reproductive toxicity includes serum concentrations of FSH, LH, testosterone and prolactin. Other indicators of central nervous system toxicity include alterations in sexual function, including libido, erection and ejaculation, although literature on occupational exposures causing sexual dysfunction in men is limited because such outcomes are difficult to assess due to a lack of objective measures and because sexual dysfunction can be attributed to, and affected by, psychological or physiological factors (Schrader, 1997).

Genetic toxicity

Genetics of male infertility While certain cytogenetic abnormalities have long been known to cause male infertility (Martin, 2008), half of male infertility cases are of unknown aetiology. A recent review by McLachlan and O'Bryan (2010) summarized current knowledge as:

- Chromosomal anomalies are found in about 7% of men with idiopathic spermatogenic failure: mainly numerical or structural anomalies in cases of azoospermia and translocations/inversions in oligozoospermia.
- Microdeletions in the azoospermia factor (AZF) region of the long arm of the Y chromosome (Yq) are the most common genetic cause of male infertility, with a prevalence of about 4% of men with <5 million sperm/ml. AZFc Yq deletions are the most common and result in low sperm production; AZFa or AZFb+c deletions usually result in failure of sperm production (no spermatozoa seen in testicular biopsies).
- Mutations of the cystic fibrosis transmembrane receptor gene (CFTR) lead to congenital absence of the vasa deferentia (CBAVD), causing azoospermia.

Until recently the main technologies for examining chromosomal abnormalities in spermatozoa were sperm karyotype analysis and fluorescent in situ hybridization (FISH) (Moosani et al., 1995; Moline et al., 2000; Carrell, 2008; Martin, 2008). However, these techniques have recently been superseded by micro-array based techniques to measure the genetic impact of environmental factors, e.g. through analysis of gene expression during spermatogenesis (Roy Choudhury et al., 2010) and genome-wide association studies of candidate genes and their polymorphisms for male fertility traits (Kosova et al., 2012; Aston and Conrad, 2013). These assays provide promising and sensitive approaches for investigating germinal and potentially heritable effects of exposures to exogenous substances, and for confirming epidemiologic observations on smaller numbers of individuals.

Sperm function: physiology, regulation and factors affecting it

Table 3 summarizes the location and possible methods for evaluating the various component processes leading to conception that involve sperm production and sperm function.

Sperm production

Spermatogenesis takes place in the germ-line cells that are embedded in the Sertoli cells of the seminiferous epithelium, which lines the seminiferous tubules of the testis. This epithelium is formed by Sertoli cells which embrace and interact with the differentiating germ cells (Clermont, 1966; Hermo et al., 2010a; Phillips et al., 2010; Oatley and Brinster, 2012). The entire process of spermatogenesis, which includes spermiogenesis, the differentiation of the round spermatid into a "testicular spermatozoon" (de Kretser, 1969; Hermo et al., 2010b,c), takes approximately 70 days to complete in man, and culminates in the release of the differentiated spermatozoa from the seminiferous epithelium or "spermiation". These "testicular spermatozoa" are not yet fully mature and must spend some 12–14 days undergoing maturation and then storage within the epididymis, although this can be shorter under some circumstances (Amann and Howards, 1980). Sperm maturation occurs in the head and body regions of the epididymis (caput epididymidis and corpus epididymidis respectively), while sperm storage is in the tail of the epididymis (cauda epididymidis) a function is shared with the "convoluted" region of the vas deferens in man (Bedford, 1994; Jones, 1999). Epididymal sperm storage is not a very efficient process, especially in man, and spermatozoa become senescent after a relatively small number of days (although this can vary between individuals). Consequently, unless ejaculation occurs every few days, the

spermatozoa in an ejaculate produced after a prolonged period of sexual abstinence might well show poor function, and many of them could be moribund or dead.

Regulation of the morphologically complex process of spermiogenesis, as well as post-testicular maturation within the epididymis and even in the female reproductive tract, involves molecular chaperones (Dun et al., 2012). This is currently an area of very active research, revealing that aberrant expression of molecular chaperones is associated with arrested spermatogenesis and sperm dysfunction (Dun et al., 2012).

A normal fertile man "in his prime" produces about 100 million spermatozoa per day (about 1000 per second) (Amann and Howards, 1980; Johnson et al., 1980).

Sperm structure and morphology

The mature spermatozoon is a highly differentiated single cell that comprises head, midpiece and tail regions (Fig. 1A), although the human sperm form is more pleiomorphic than in almost any other species (Fig. 1B). The human sperm head is a flattened pear shape, with the point anteriormost, approximately 5 µm long, 3.5 µm wide and 1.5 µm thick. In plan view (after settling or drying down onto a flat surface) it has a smooth oval outline within which small "vacuoles" are seen quite commonly in stained preparations; the presence of many or large vacuoles being considered abnormal (Fig. 2A,B). These "vacuoles" can be either small clear spaces within the condensed chromatin of the sperm nucleus that are not membrane bound ("nuclear vacuoles", more correctly "nuclear spaces"), or else depressions in the surface of the sperm head, often with a raised ridge around their rim, disrupting the underlying structures. "Craters" are clearly different from nuclear vacuoles, and are believed to have been created by protrusions of the Sertoli cell into the round spermatid during spermiogenesis.

In recent years these "nuclear vacuoles" have been of great interest in regard to selecting spermatozoa for an advanced version of intracytoplasmic sperm injection (ICSI) known as "IMSI" (intracytoplasmic morphologically selected sperm injection), based on sperm normality as defined by "MSOME" (motile sperm organelle morphology examination), i.e. the absence of "nuclear vacuoles" (Berkovitz et al., 2006; Kacem et al., 2010). While it has become clear that it is spermatozoa without "craters" (which are often described as structural defects of the acrosome, and can disappear following induction of the acrosome reaction (Montjean et al., 2012)) that are being selected, the functional damage appears to be abnormal chromatin packaging leading to sperm DNA damage (Oliveira et al., 2010; Boitrelle et al., 2011; Perdrix et al., 2011; Franco et al., 2012), and also aneuploidy (Perdrix et al., 2011).

The sperm nuclear envelope is unique in that it does not have any nuclear pores except in the very limited region between the posterior ring, which marks the posterior boundary of the post-acrosomal sheath (a wide belt-like structure that wraps around most of the posterior 35–40% of the sperm head) and the basal plate, a thickened structure of the nuclear envelope that acts to create the implantation fossa for the connecting piece of the neck region, where the sperm tail "articulates" with the head (Fig. 2A,B). Human spermatozoa in particular often have "redundant nuclear envelope" that hangs like a folded curtain from this region, enclosing a small volume of nucleoplasm (Pedersen, 1969), which has recently been identified as a probable calcium store important for sperm hyperactivation (Ho and Suarez, 2003; Chang and Suarez, 2010). Zinc also appears to have a role in the sperm-tail connection since its extraction promotes tail detachment (Björndahl and Kvist, 1982). The post-acrosomal sheath is a vitally important structure during fertilization, being where the fertilizing spermatozoon first binds to the oolemma, and the posterior ring serves as an O-ring seal around the posterior of the sperm head, effectively separating the cell into head and tail compartments (Gadella et al., 2008).

The acrosome, a cap-like structure derived from the spermatocyte Golgi apparatus, lies directly under the plasma membrane in the anterior 40–70% of the sperm head and is closely associated with the nuclear envelope, being separated from it by a minimal sub-acrosomal space. The acrosome comprises a larger anterior "cap" portion, which will undergo the acrosome reaction (see below), and a posterior "equatorial segment" region. Although an integral part of the acrosome, the equatorial segment is distinct from the cap as it does not participate in the acrosome reaction. Indeed, it must remain intact as it is the region where fusion between the spermatozoon and the oocyte is initiated during fertilization.

The midpiece region of the spermatozoon (typically 5–7 µm long) actually comprises the connecting structures of the neck region and the anterior part of the sperm tail, where the axial filament complex of the flagellum is enveloped by a double helical spiral of elongated mitochondria (Fig. 2C). The connecting piece is made up of quite rigid glycoprotein "striated column" structures, and is attached to the basal plate region of the sperm nuclear envelope. Inside the anterior part of the striated columns is buried the proximal centriole, the distal centriole having served as the origin of the 9+2 structures of the sperm flagellum but being lost in the mature spermatozoa of the great majority of Eutheria. In most Eutheria (but notably excluding the mouse), the sperm proximal centriole will be the microtubule organizing centre for the first cleavage division of the fertilized oocyte.

The sperm tail is built upon the typical 9+2 microtubular structure of cilia and flagella, but with an additional set of outer "dense"

fibres that are generally accepted to serve as an elastic element to increase the power generation of the beating tail (evolutionarily, they are generally present only in spermatozoa from species with internal fertilization). Hence for much of its length (50–60 µm in human spermatozoa), including the midpiece, the sperm flagellum is actually a 9+9+2 structure (Fig. 2C,D). Posterior to the midpiece (whose posterior limit is marked by the "annulus") the axial filament complex is surrounded by a different structure, the fibrous sheath, made up of a pair of longitudinal columns to which are attached numerous C-shaped, inter-branching, rib-like elements (Fig. 2D). The various dense fibres terminate at different (but relatively specific) positions along the tail, and their asymmetric termination is believed to be responsible for the creation of a third-dimensional component in the otherwise planar wave generated by the beating of the 9+2 structured sperm flagellum (Woolley, 1979; Serres et al., 1983). This 3-D "twisted plane" component is essential for achieving progression of the swimming spermatozoa within a low Reynolds number system (Smith et al., 2009), where the relationship between the spermatozoon and its environment is one of very high viscosity, even "stickiness" ($\boldsymbol{R} \sim 10^{-4}$) , due to the absolute scale of magnitude at which it exists – it is massively different to the perception of a human being or a fish swimming in water ($\boldsymbol{R} \sim 10^{4}$) (Purcell, 1977; Mortimer, 2002; Kirkman-Brown and Smith, 2011). In the most distal part of the sperm tail, the terminal segment, only the basic 9+2 structure remains.

The surface of the spermatozoon is covered by a dense carbohydrate coat, the glycocalyx, which is formed from secretions of the epididymis. The glycocalyx protects the spermatozoa within the male tract, as well as during transit through the female tract where it plays a key role in regulating sperm function, including immunoprotection (Schröter et al., 1999; Tollner et al., 2011).

Sperm morphology at the light microscope level: Assessments of human sperm morphology at the light microscope level, typically using fixed and stained smears made from liquefied semen, is a fundamental part of the basic semen analysis (Mortimer, 1994; Coetzee et al., 1998; Björndahl et al., 2010; World Health Organization, 2010). Several schemes for assessing and classifying human sperm morphology have been described, but the most common ones in current use are those promoted by the ESHRE Special Interest Group in Andrology (Björndahl et al., 2010) and the WHO (World Health Organization, 2010) – both of which are based on the Tygerberg Strict Criteria (Kruger et al., 1986; Menkveld et al., 1990) – as well as the David system that is widely used in French-speaking countries (David et al., 1975; Auger, 2010).

Modern concepts of sperm normality are based on studies of spermatozoa recovered from cervical mucus post-coitally (Menkveld et al., 1990; Mortimer and Menkveld, 2001) and supported by observations of spermatozoa that bind to the zona pellucida in vitro (Liu and Baker, 1992).

There is growing opinion that the term "normal" should be replaced by "ideal" or "typical", and that the % ideal spermatozoa – which is usually very low – be supplemented by additional information on the degree of malformation of the abnormal spermatozoa, which is a negative predictor of biological function (Auger, 2010; Björndahl et al., 2010).

In general terms, a "typical" or "ideal" human spermatozoon has an oval head form with a smooth contour and a clearly visible and well defined acrosome. The tail is inserted symmetrically without any abnormalities of the neck/midpiece and tail region, and there are no cytoplasmic residues in the neck/midpiece or tail regions. The sperm head is between 3.0-5.0 µm in length and 2.0-3.0 µm in width, with the acrosome covering 30%–60% of its anterior region. The midpiece is no longer the 1.5× the length of a typical head, and about 1 µm thick. The tail is 45-50 µm long with no sharp bends or kinks. Spermatozoa with any features that appear "borderline" or slightly atypical are considered to be atypical (Björndahl et al., 2010).

Sperm motility

As spermatozoa mature in the epididymis they gain the capacity for motility, but do not express motility until they are "activated" at ejaculation. Spermatozoa removed directly from the testis are capable, once washed into culture medium, of only twitching motility, although a few progressively motile spermatozoa can sometimes be found (Björndahl et al., 2010). This twitching motility can be enhanced with phosphodiesterase inhibitors such as pentoxifylline (see *Factors affecting male reproductive capacity – Pharmaceuticals – Phosphodiesterase inhibitors*, above). The percentage of testicular spermatozoa exhibiting motility may increase with overnight incubation (Björndahl et al., 2010). Progressive motility requires ATP production, by both anaerobic glycolysis in the sperm tail and oxidative phosphorylation in the mitochondria of the sperm midpiece (Piomboni et al., 2012). Many compounds are known to inhibit these processes, and even more can cause outright sperm cell death by permeabilizing the sperm membrane, preventing normal membrane function, or inhibiting other critical processes.

Progressive motility is normally an attribute of, at a minimum, around half of the spermatozoa in an ejaculate; many spermatozoa in semen exhibit non-progressive motility, meaning that their tails are beating but the cell is not moving from one place to another; other spermatozoa are non-motile (Mortimer, 1994; Mortimer ST, 1997; Björndahl et al., 2010; World Health Organization, 2010). Sperm motility is induced by beating of the sperm flagellum, with waves being initiated at the anterior end of the tail, inside the midpiece, where it joins the connecting piece (reviews: Mortimer, 1997; Turner, 2006; Lindemann and Lesich, 2010; Woolley, 2010; Inaba, 2011). Careful analysis of swimming spermatozoa has revealed that each beat can be defined in terms of beat initiation, wave development, and wave propagation. During the wave development phase the curvature of the tail increases, and is

dependent on intracellular Ca^{2+} concentration – so a delay in wave propagation will result in a larger amplitude wave being sent down the tail, generating greater propulsive force (Mortimer et al., 1997; Olson et al., 2010; Ishijima, 2011). Larger amplitude waves lead to higher sperm velocity as well as greater lateral displacement of the sperm head – a pattern of movement that is essential for effective penetration into, and migration within, cervical mucus, and failure to develop such motility has been established as a cause of infertility (Feneux et al., 1985). The study of sperm motility patterns is termed *kinematics* (Fig. 3) and has been greatly facilitated since the development of computer-aided sperm analysis technology ("CASA"; Mortimer D, 1994; Mortimer ST, 1997). The actual human sperm trajectory in semen in three-dimensional space is an ovoid helix with a radius of about 0.5–3.0 μm; a significant majority of spermatozoa (*ca.* 90%) preferring right-handed helices over left-handed ones (Su et al., 2012).

Sperm survival / longevity: Spermatozoa have a finite functional lifespan, a maximum of a few days within the human female reproductive tract (reviews: Mortimer, 1983, 1995b). Loss of motility is the most obvious indication of cell death, although a non-motile spermatozoon might still be alive in terms of membrane integrity (Björndahl et al., 2003). Studies of human spermatozoa removed from the cervix at different times after insemination demonstrated that spermatozoa lose their ability to acrosome react and fuse with an oocyte in about 2 days, but remain motile for another day or so (Zinaman et al., 1989).

As noted already, oxidation of docosahexanoic acid in the sperm plasma membrane is the major factor that determines the motile lifespan of spermatozoa *in vitro* (Aitken and Clarkson, 1987), as well as membrane damage and DNA oxidation (Fraga et al., 1991, 1996).

Because of its ease of measurement, sperm motility is often used by clinical laboratories as a *sperm survival assay* as part of the quality control processes for plastic- or glass-ware and culture media (Claassens et al., 2000; Björndahl et al., 2010). Residual chemicals, detergents, plasticizers and endotoxins or other biological toxicants adversely affect sperm survival. As a clinical test, a survival assay is a measure of sperm "hardiness", although particular assay conditions can influence the outcome (e.g. a highly stimulatory medium can promote high ATP generation or acrosome reactions, either of which will lead to a shortened lifespan for spermatozoa). Human sperm motility after an overnight incubation in IVF medium is a major predictive component of pregnancy after intra-uterine insemination (Branigan et al., 1999).

Sperm transfer: ejaculation, the ejaculate and impregnation

Ejaculation typically requires erection of the penis, and this is essential for impregnation of the female via intercourse. Erectile dysfunction can therefore be a serious cause of subfertility, although many types of erectile dysfunction can now be treated using inhibitors of phosphodiesterase type 5, and electro-ejaculation can also be performed in extreme cases, e.g. those involving many types of spinal cord injury (van Ahlen and Kliesch, 2010). However, it is not known to what extent electroejaculation mimics the normal ejaculation process, where spermatozoa are transferred from the cauda epididymides and "convoluted vas" and mixed with the secretion from the prostate gland before being pumped through the urethra and emitted to the exterior through the meatus at the tip of the glans penis. This initial "sperm rich" fraction, which makes up about one-third of the total ejaculate volume in man, is followed by the secretion from the seminal vesicles (Mann, 1964; Björndahl et al., 2010).

In the *in vivo* situation, i.e. during intercourse, the sperm-rich fraction of the ejaculate is deposited around the external os of the uterine cervix, from which the mucous secretion of the cervical epithelium (cervical mucus) protrudes. This may allow prompt migration of spermatozoa into the protective environment of the cervical mucus, away from the vaginal environment, which is quite acidic and therefore hostile to spermatozoa. The seminal vesicular secretion coagulates upon emission, creating a "copulatory plug" in some species, e.g. rodents and many primates, that prevents another male from impregnating a female for some time, creating a competitive advantage for the first male's spermatozoa (Mann, 1964). In man, coagulation of the vesicular secretion is merely a vestigial remnant of the copulatory plug. However, when ejaculates are collected for laboratory testing, either by masturbation, withdrawal (*coitus interruptus*), or using a special non-toxic condom, all fractions of the ejaculate are intermixed, with the intermixed secretions of the male accessory glands constituting the seminal plasma. Hence the spermatozoa are trapped within the coagulum until the proteins that caused coagulation can be broken down by proteolytic enzymes present in the prostatic fluid component. In strict physiological terms the combined ejaculate can be considered an artefact, and has caused some confusion in our understanding of sperm physiology *in vivo*.

At the time of ejaculation, Eutherian spermatozoa are not capable of achieving fertilization due to the presence of "decapacitation factors" present in the seminal plasma, whose physiological purpose is to maintain the spermatozoa in a stable state during epididymal storage (Leahy and Gadella, 2011). One mechanism whereby decapacitation factors achieve their function is through stabilizing the sperm plasma membrane by maintaining a particular cholesterol:phospholipid ratio.

Sperm transport through the female reproductive tract

Overview: Among Eutheria there are numerous variations in mechanisms for sperm deposition within the female reproductive

tract and their subsequent transport to, and storage at, the site of fertilization (Mortimer, 1983, 1995b; Suarez and Pacey, 2006; Ikawa et al., 2010). Basically, there are two major strategies: "uterine" and "vaginal" insemination – with humans employing the latter.

Sperm-cervical mucus interaction: Once deposited around the external os, spermatozoa must penetrate into the mucus secreted by the uterine cervix and which occludes the cervical canal. The cervical mucus is only receptive to spermatozoa around mid-cycle (Mortimer, 1983, 1994, 1995b; Björndahl et al., 2010). Mid-cycle (periovulatory) cervical mucus is unique in its water content and molecular arrangement, easily tested by its "Spinnbarkeit" and "ferning" properties, with its secretion being under the control of estrogen. Penetration into, and migration within, cervical mucus depends on effective progressive motility (Mortimer, 1983; Feneux et al., 1985); the older "insuck" theory associated with female orgasm is no longer afforded any real credibility (Mortimer, 1983; 1995b). Cervical mucus may harbour anti-sperm antibodies or other poorly defined molecules that immobilize spermatozoa.

There is no physiological evidence for a "cervical sperm reservoir" as described in older textbooks, nor is there evidence to support relationships between coital position and fecundity, coital practices and the gender of resulting offspring, or between female orgasm and fertility (Levin, 2011). As spermatozoa migrate into cervical mucus they escape from the decapacitation factors-rich seminal environment and the process of "capacitation" commences. Spermatozoa must swim through the column of cervical mucus that occludes the uterine cervix.

Sperm migration through the uterus and oviducts: Once within the uterine lumen (which, in nulliparous women, is really only a virtual cavity, with only a thin film of uterine fluid separating the quite closely apposed anterior and posterior walls of the uterus). The most likely explanation for the transfer of spermatozoa from the internal os of the cervix to the utero-tubal junctions is their being spread by segmental contractions of the uterine wall, and migration through the utero-tubal junction appears to depend on sperm motility (Mortimer, 1983, 1995b; Suarez and Pacey, 2006; Ikawa et al., 2010). In humans, the isthmus region of the oviduct (Fallopian tube) serves as the site of physiological sperm storage, although solid evidence regarding this function has proven difficult to obtain in women (Williams et al., 1993; Mortimer, 1995b). The isthmic environment is capable of regulating sperm function during their storage there, with spermatozoa usually interacting intimately with the lining of the isthmus. During isthmic sperm storage, the completion of capacitation seems to be suppressed, and release from the isthmic sperm reservoir seems to be regulated via a portal blood supply linking the venous drainage from the ovary to the arterial supply of the ipsilateral oviduct (Suarez and Pacey, 2006; Suarez, 2007, 2008; Holt and Fazeli, 2010).

Sperm seem to continue to bind to oviductal epithelium after leaving the isthmic reservoir, and motility is crucial in their migration to the site of fertilization in the oviductal ampulla (Chang and Suarez, 2012). Regional gradations in oviduct fluid viscosity, perhaps combined with temperature gradients, could interact with sperm flagellar activity to reveal subtle differences in sperm functional potential (Hunter et al., 2011) and create environments for sperm selection (Kirkman-Brown and Smith, 2011).

Capacitation and hyperactivation

Although ***sperm capacitation*** was discovered 60 years ago (Chang, 1951; Austin, 1952), and is defined as resulting in the spermatozoon having acquired the capacity to undergo the acrosome reaction and fertilize an oocyte, its molecular mechanisms are still not fully understood (Yanagimachi, 1994; Fraser, 1998; Bedford, 2004; De Jonge, 2005; Salicioni et al., 2007). Several changes take place in different regions of the spermatozoon, including changes in the sperm plasma membrane that involve the loss of decapacitation factors and cholesterol (Leahy and Gadella, 2011), phosphorylation of tyrosine residues in the outer dense fibres and fibrous sheath of the tail (allowing the tail to become more flexible and exhibit different patterns of beating to those seen in ejaculate or "seminal" spermatozoa) (Yunes et al., 2003; Mariappa et al., 2006), the ability to generate calcium oscillations (Aitken and McLaughlin, 2007), and changes in the acrosome that prepare it to undergo the acrosome reaction (Yanagimachi, 2011). Capacitation is an essential requirement for fertilization, both *in vivo* and *in vitro* for IVF, and it must be adequately supported by any culture medium that is to be used in an IVF system.

The sperm head plasma membrane undergoes major reorganization, notably the formation of lipid ordered microdomains (lipid rafts) during capacitation with the dynamic redistribution of membrane molecules over its surface that create functionally heterogeneous regions such as the area overlying the acrosomal cap which participates in the acrosome reaction (Gadella et al., 2008; Leahy and Gadella, 2011). Capacitation can be promoted by substances such as albumin or minor components such as cholesteryl ester transfer protein (CETP; Muller and Ravnik 1995; Ravnik et al., 1995), and bicarbonate (Gadella and Van Gestel, 2003; Visconti et al., 2011), and even to some degree over-ridden by powerful agonists such as ß-cyclodextrins, which sequester cholesterol (Visconti et al., 1999), or by-passed by calcium ionophores which can trigger the acrosome reaction more-or-less directly (Bailey, 2010). Capacitation is regulated by increased intracellular pH (pH_i having been maintained at a slightly acidic level during epididymal storage) via the Hv1 voltage-sensitive proton channel (Lishko et al., 2010; Florman et al., 2010).

Hyperactivated motility is expressed as a concomitant of capacitation: physiologically during the passage of spermatozoa through the upper parts of the female reproductive tract to the site of fertilization in the oviductal ampulla, or under capacitating

conditions *in vitro*. It is characterized by the development of high amplitude flagellar waves, which causes a "whiplash-like" movement of the head in free-swimming spermatozoa (Yanagimachi, 1994; Mortimer ST, 1997). The vigorous beating pattern serves to dislodge spermatozoa that have bound to the oviductal epithelium, and later, to help drive the fertilizing spermatozoon through the zona pellucida.

Hyperactivation depends on increased flexibility of the tail structures, combined with increased $[Ca^{2+}]_i$, presumably originating from a calcium store located in the sperm neck and midpiece (Costello et al., 2009; Buffone et al., 2012), and a delay in wave propagation, results in beats of greatly increased amplitude (Fig. 3B). If the delay in wave propagation is increased further then the curvature developed in the proximal part of the tail can cause the sperm head to turn back on itself, so that when the wave is propagated (at much increased velocity in hyperactivated spermatozoa) the spermatozoon "thrashes", displaying a non-progressive "whiplash" pattern of movement that is generally characteristic of hyperactivated Eutherian spermatozoa examined *in vitro* (Yanagimachi, 1970; Mortimer et al., 1997), and can also been seen *in vivo* in some species where suitable observations can be made (Yanagimachi, 1970).

Hyperactivated spermatozoa show poor ability to traverse the utero-tubal junction (Shalgi et al., 1992), and hence premature sperm capacitation (e.g. in sperm preparations intended for intra-uterine insemination) could be severely disadvantageous, and preclude proper sperm transport to the site of fertilization.

In vitro, the proportion of spermatozoa with hyperactivated motility can be quantified using computer-aided motility analysis (Mortimer ST, 1997; Björndahl et al., 2010). True hyperactivated motility does not occur in seminal plasma. While a similar pattern of beating can be seen following ROS-induced damage (de Lamirande et al., 1997), it fails to meet proper kinematic criteria for hyperactivation (because tyrosine phosphorylation has not occurred and the axial filament complex is not yet in its "capacitated" state), and must be seen as a pathological event that could lead to impaired fertility.

The role of calcium ion dynamics in the initiation and maintenance of hyperactivated motility involves CatSper (cation channel of sperm) a pH-activated weakly voltage-dependent calcium channel (Qi et al., 2007; Publicover et al., 2008; Olson et al., 2010; Brenker et al., 2012; Lishko et al., 2012), whose action can be integrated with hydrodynamics and flagellar modelling to create a regulatory model for sperm hyperactivation (Olson et al., 2011a,b). CatSper also mediates progesterone-induced Ca^{2+} influx into human spermatozoa (Strünker et al., 2011), thereby serving as a non-genomic progesterone receptor in spermatozoa that can modulate $[Ca^{2+}]_i$ as the basis for chemotaxis (Publicover et al., 2008; Yoshida and Yoshida, 2011). Hyperactivation and chemotaxis have been combined into models for how spermatozoa locate the oocyte within the three dimensional space of the oviduct (Guerrero et al., 2011),

Sperm-oocyte interaction, including the physiological acrosome reaction

Cumulus penetration: After reaching the site of fertilization in the ampulla of the oviduct (Fallopian tube), the fertilizing spermatozoon must migrate through the cumulus mass and corona radiata cells surrounding the oocyte (Ikawa et al., 2010; Kirkman-Brown and Smith, 2011). Although the matrix of the cumulus mass has a very high hyaluronate component, the traditional view that this migration was helped by hyaluronidase from the acrosome is now considered circumspect, it now being unclear whether what was thought to be the enzyme hyaluronidase actually has catalytic activity (Kang et al., 2010).

Sperm-zona pellucida binding: The conventional view is that upon reaching the zona pellucida the spermatozoon binds, in a more-or-less species-specific way at least partly dependent on lectin-like (sugar-protein) interactions, to putative sperm receptors which then induce the physiological acrosome reaction (Yanagimachi, 1994; Visconti and Florman, 2010). Although recent research in mice suggests the physiological acrosome reaction is probably initiated prior to binding to the zona pellucida (Jin et al., 2011; Yanagimachi, 2011), in humans most of the spermatozoa recently bound to the zona pellucida are acrosome-intact (Cross et al., 1988), and acrosome-reacted spermatozoa are unable to bind to the zona pellucida (Liu et al., 2006).

Acrosome reaction: The acrosome reaction is a complex event, involving extrinsic receptors, signal transduction, influx of calcium, membrane fusion and vesiculation with loss of parts of the plasma membrane and outer acrosomal membrane, and activation and release of enzymes from the acrosome (Yanagimachi, 2011). Prevention or disruption of any of these steps can block the event, and thus prevent fertilization. In human spermatozoa it is believed that the acrosome reaction is induced following binding to a putative sperm receptor integral to the zona pellucida, most probably zona pellucida glycoprotein 3 (ZP3) and proceeds via a still poorly characterized cascade of intracellular biochemical pathways (Visconti, 2009; Visconti and Florman, 2010). However, substantial elevation of $[Ca^{2+}]_i$ is induced, perhaps involving a store in the acrosomal region (Costello et al., 2009; Buffone et al, 2012), and a Ca^{2+}-dependent Cl^- current can be identified during acrosome reactions induced by a recombinant ZP3 (rhZP3) molecule in vitro (Orta et al., 2012).

The *normal acrosome reaction* (Fig. 2A,B) involves dissociation of the tightly packaged pro-enzymes stored inside the acrosome (mostly pro-acrosin), followed by localized fusions between the sperm plasma membrane and the outer acrosomal membrane of the cap region of the acrosome creating openings through which enzymes are released (Zanetti and Mayorga, 2009). While

acrosin may act to soften the glycoprotein matrix of the zona pellucida, the fertilizing spermatozoon does not simply digest its way through the zona: its hyperactivated motility generates propulsive force 10× greater than that required to break covalent bonds (Drobnis et al., 1988), hence it pushes its way through the softened substance of the zona. Ultimately these membranes are lost, leaving an acrosome-reacted spermatozoon whose anterior exterior surface is the inner acrosomal membrane (Yanagimachi, 1994; Ikawa et al., 2010). In a normally acrosome-reacted spermatozoon the equatorial segment of the acrosome must remain intact to initiate fusion between the sperm and oocyte plasma membranes (spermalemma and oolemma) during fertilization; without the equatorial segment a spermatozoon cannot achieve fertilization. Under *in vivo* conditions the acrosome-reacted spermatozoon is held in place by virtue of being embedded within the corona radiata and cumulus mass, and so is ready to move into the next stage of its penetration of the oocyte vestments.

Capacitated spermatozoa are in a highly labile state, and can also initiate *acrosome loss* via a *false acrosome reaction* in response to many physical and chemical triggers (e.g. elevated pH: Mortimer, 1995a). Premature loss of the acrosome (Tesarik, 1989; ESHRE Andrology Special Interest Group, 1996), as a result of either a normal or false acrosome reaction before the spermatozoa reach the zona pellucida prevents their binding to it (Liu et al., 2006) – thereby impairing or perhaps preventing fertilization.

Sperm-oolemma binding and sperm-oocyte fusion: Once through the zona pellucida, the spermatozoon makes contact with the oolemma and a specific binding process occurs between the oolemma and the surface of the posterior sheath region of the spermatozoon, although the molecular details of this are still largely unresolved (Ikawa et al., 2010). Based on old microcinematographic studies (RJ Blandau, Seattle, WA, USA) sperm motility ceases at this point and the remainder of the process of sperm incorporation into the oocyte is effected by the oocyte. Sperm-oocyte fusion is initiated between the remaining sperm plasma membrane over the equatorial segment and the oolemma, and the entire spermatozoon, including the midpiece and sperm tail structures, is incorporated into the oocyte and disassembled (Yanagimachi, 1994; Sutovsky and Schatten, 2000).

The sperm mitochondria, which have been tagged with ubiquitin, are destroyed, although remnants may be identifiable at the ultrastructural level during the first one or two cleavage divisions (Sutovsky et al., 2000; Baska et al., 2008). The 9+9+2 axial filament complex and the fibrous sheath of the sperm tail are all disassembled within the ooplasm. The sperm plasma membrane remains as a "patch" in the oolemma, it only enters the ooplasm during ICSI (intra-cytoplasmic sperm injection), and there is evidence that ROS-based oxidation of its phospholipid and cholesterol content (the latter is especially high, since spermatozoa used for ICSI are typically uncapacitated) can adversely affect embryonic development (Tateno and Kamiguchi, 2007).

Sperm contributions at fertilization: The fertilizing spermatozoon brings three essential components to the oocyte: the *male haploid nucleus*, *centrosome* (centriole), and an *egg activating factor* (Sutovsky and Schatten, 2000; Barroso et al., 2009), any defects in any of these three elements can result in fertilization failure. After the fertilizing spermatozoon fuses with the oolemma and is drawn into the cytoplasm of the oocyte, the sperm nucleus must decondense to form the male pronucleus. In some cases, the decondensation step occurs too early or not at all and can result in failed fertilization, even following ICSI (Sakkas et al., 1996; Esterhuizen et al., 2002; Björndahl and Kvist, 2010). After the male and female pronuclei fuse, their respective chromosomes must line up correctly and subsequently separate for the embryo's first cell division. In most Eutheria this division is organized by the centriole brought in by the spermatozoon (Schatten, 1994; Sathananthan et al., 1996, 2006). Damaged centrioles will not prevent fertilization, but will lead to the early demise of the embryo. The third critical contribution is a signalling molecule, a sperm-specific phospholipase, PLCζ (Kashir et al., 2010; Ramadan et al., 2012), that triggers calcium oscillations and induces the resumption of the second meiotic division (Ajduk et al., 2008).

Syngamy and early embryonic development

After the sperm nucleus has been incorporated, its nuclear envelope is lost and a new one forms from oocyte-derived components as the sperm chromatin is decondensing and its protamines are replaced by histones. During this process there is opportunity for some repair to sperm DNA damage, but it is limited by time (De Boer et al., 2009). Once this process is complete the structure is known as the male pronucleus, which then merges with the female pronucleus at syngamy, marking the completion of the fertilization process and the creation of a new genetic individual.

Development of the early embryo is controlled by a pool of mRNAs that was established in the ooplasm during the final stages of oocyte maturation inside the Graafian follicle (Gosden, 2002; Krisher, 2004). Timing of activation of the human embryonic genome (transcription) has generally been believed to begin between the 4- and 8-cell stage, and translation at the 8-cell stage. However, a recent report has described the presence of waves of transcriptional activity starting as early as the 2-cell stage (as well as waves of degradation of maternal mRNA), although it remains unclear when these mRNAs are translated (Vassena et al., 2011). Hence the embryonic genome is responsible for development beyond the 8-cell stage, i.e. from Day 3 (Braude et al., 1988; Wong et al., 2010).

The paternal DNA is highly susceptible to fragmentation caused by chemotherapeutic drugs, radiation, and other mechanisms that increase oxidative stress (Barratt et al., 2010). Fragmented sperm DNA may or may not be able to be repaired by the oocyte or

early embryo, depending on the extent of damage. Also, small fragments of DNA that result from "double-double" breaks might be integrated at the wrong place or inverted, resulting in spontaneous "mutations" that could lead to serious childhood disorders. A number of childhood diseases have already been linked to paternal age, and sperm DNA damage increases with age (Singh et al., 2003). There are numerous recent reviews on the role of sperm DNA damage in male infertility (e.g. Barratt et al., 2010; Lewis and Simon, 2010; Pacey, 2010; Gharagozloo and Aitken, 2011), but although many published studies claim that sperm DNA damage / fragmentation has been assessed, the measurement methods are only indirect – it remains unclear whether some of these "assays" detect actual endogenous DNA damage or just increased vulnerability of the sperm DNA (Barratt et al., 2010). Nonetheless, embryos with massively damaged sperm-derived haploid genomes usually appear perfectly normal up until Day 3 of development, but typically have extremely poor capacity to form normal blastocysts and implant (Tesarik, 2005; Barroso et al., 2009; Seli and Sakkas, 2009; Sakkas and Alvarez, 2010).

Assessments of sperm toxicity

Experimental and biological artefacts

There are two major sources of artefacts in many *in vitro* studies on sperm physiology: the impact of viscosity on sperm motility and the collection of the ejaculate as a single specimen.

Viscosity: Changes in the viscosity of the environment in which spermatozoa are swimming will affect their movement, and hence analyses of their progressive motility and kinematics. A non-engineer's perceptions of hydrodynamics are based upon human perceptions of aquatic organisms, and their simple extrapolation to spermatozoa will lead to erroneous concepts unless the Reynolds number (***R***: the ratio of the inertial forces to the viscous forces) of the system is taken into account (Purcell, 1977; Mortimer, 2002; Smith et al., 2009, Gadêlha et al., 2010). Sperm motility is far more complex than simple consideration of the "percentage motile" or "progressive" spermatozoa, and robust analysis of sperm movement patterns (sperm "kinematics") requires consideration of the interaction between flagellar beating patterns and the physical nature of the environment in which spermatozoa exist. Consequently, any studies in which the viscosity of products (e.g. sperm washing or culture medium or lubricant) is modified must include proper controls to avoid artefacts, which can easily be misconstrued as impairments (or enhancements) to sperm motility, and hence perceived functional ability.

The collected ejaculate: This artefactual situation, brought about by collecting the human ejaculate as a single specimen, results in several abnormal (non-physiological) circumstances that can have major impacts on spermatozoa (e.g. spermatozoa in the second fraction of ejaculates in cases of asthenozoospermia and teratozoospermia show elevated levels of sperm DNA fragmentation (Kumar et al., 2011). However, for many practical reasons it is difficult to avoid the routine collection of the whole ejaculate in a single container, and this practice will most likely continue unless some diagnostic or therapeutic advantage to examining or separating spermatozoa from the first fraction of a carefully collected split ejaculate has been established. Hence collecting the complete human ejaculate for analysis remains the de facto standard for the primary research tool for studying the effects of toxicants on the male reproductive system (Cohn et al., 2002; Brazil et al., 2004a,b).

Exposure of spermatozoa to seminal vesicular secretion: Spermatozoa initially exposed to seminal vesicular fluid rather than prostatic fluid are less motile and show impaired survival (Lindholmer, 1973). Furthermore, due to extraction of nuclear zinc by seminal vesicle fluid there is an initial increased accessibility of the DNA followed by a rapid super-stabilization of the chromatin. Increased DNA accessibility constitutes an opportunity for increased DNA damage, especially in an oxidative environment such as the seminal vesicular fluid. Subsequently, after spermatozoa have been exposed to the seminal plasma for more than a few minutes, access to the DNA is reduced due to excessive disulphide bond formation (Björndahl and Kvist, 1985; Kvist et al., 1985), a phenomenon that can influence sperm chromatin damage test results by (a) concealing DNA damage due to reduced access to the DNA (false negative results) and (b) inducing more damage *in vitro* beyond what already existed *in vivo* (false positive results). Thus, a sperm-safe product should take into consideration possible effects on sperm chromatin structural stability as well as the risk for oxidative damage.

Prolonged exposure to seminal plasma: Eutherian ejaculated spermatozoa are prevented from undergoing capacitation by decapacitation factors(s) present in the seminal plasma. Seminal plasma also contains one or more factors to which prolonged exposure adversely affect sperm function, including the ability to penetrate cervical mucus, undergo the acrosome reaction *in vitro* and the fertilization process generally (Yanagimachi, 1994; Mortimer, 2000). Based on studies using the zona-free hamster oocyte penetration assay, exposure of human spermatozoa to seminal plasma for more than 30 minutes after ejaculation can permanently diminish their fertilizing capacity (Rogers et al., 1983), and contamination of prepared sperm populations with as little as 0.01% (v/v) of seminal plasma (i.e. a 1:10,000 dilution) can decrease their fertilizing capacity (Kanwar et al., 1979). As discussed already, the exposure of human spermatozoa to the hypertonic conditions in liquefied ejaculates can be considered unphysiological and might have significant implications for their subsequent handling and processing. Clearly spermatozoa for

clinical procedures (e.g. IUI or IVF) or laboratory tests of sperm fertilizing ability must be removed from the seminal plasma as soon as possible after ejaculation (but which can be delayed to allow for normal liquefaction at 37°C), and why poor sperm washing protocols could adversely affect sperm functional potential as a result of carry-over of seminal plasma.

Human sperm motility and vitality

In vitro assessment of sperm progression is the oldest way of assessing sperm function (Botella-Llusia, 1956) and for testing substances for adverse affects on sperm function, e.g. the transmembrane migration test (Hong et al., 1981a). Semen analysis sperm motility assessments have long been used in studies on reproductive toxicology (Wyrobek et al., 1983b; Schrader et al., 1991; Cohn et al., 2002), and the need for standardized training, robust protocols and quality control are well-recognized (Brazil et al., 2004a,b).

Visual assessments of sperm motility under a microscope are notoriously subjective, and without rigorous training most observers can only provide "guesstimates" as to even the proportion of motility, or progressively motile, spermatozoa. This problem has been known for 60 years (MacLeod and Gold, 1951), but it can be eliminated provided that thorough, goal-orientated training using qualified reference materials is undertaken (Mortimer, 1994). The issue of the quality of sperm progressive motility has also been recognized since 1951, and it can also be addressed via thorough training (Barratt et al., 2011). Being able to determine the proportion of spermatozoa that show rapid progression (defined as those with a space-gaining velocity of at least 25 µm/s, at 37°C) identifies the sub-population of spermatozoa that are biologically – and hence clinically – important (Barratt et al., 2011).

Computer-aided sperm analysis (CASA) technology is still unable to provide reliable values for either total sperm concentration or the proportions of motile or progressively motile spermatozoa in routine practice without a substantial uncertainty of measurement (Mortimer et al., 1995; ESHRE Andrology Special Interest Group, 1998; Mortimer and Mortimer, 1998). However, CASA can be used reliably to quantify specific sub-populations of spermatozoa with functional potential, e.g. those that show the correct kinematics for penetrating into cervical mucus (ESHRE Andrology Special Interest Group, 1998; Mortimer ST, 2000).

When analyzing sperm motility as a marker of spermotoxicity an assay can be either a single time endpoint or else a longitudinal study over a period of time that is commensurate with physiology, e.g. the detection of spermotoxicity to spermatozoa in seminal plasma of supposedly "non-spermicidal" lubricant and ultrasound gels used in reproductive medicine (Agarwal et al., 2008; Vargas et al., 2011). For spermatozoa in semen this means no more than a short-term incubation period since spermatozoa that migrate from the ejaculate into cervical mucus *in vivo* do so within a very short period of time, making seminal plasma a non-physiological environment for human spermatozoa beyond that time window (MacLeod and Gold, 1951; Mortimer, 1995b); longer incubation periods, e.g. several hours, can only be seen as a sort of unphysiological "stress test". Using an elevated incubation temperature, e.g. 39°C, only exacerbates this issue. It should also be noted that the very high protein content of seminal plasma can protect the spermatozoa against certain types of spermotoxicity.

The most common, and physiologically valid, sperm motility assays are "sperm survival assays" whereby spermatozoa are separated from liquefied semen and resuspended in a physiological culture medium capable of supporting their motility. Such assays serve two purposes: (a) a bioassay for testing culture media and contact materials in an ART laboratory (Claasens et al., 2000; De Jonge et al., 2003); and (b) to evaluate the longevity of a man's spermatozoa (Coccia et al., 1997). While well-validated protocols are available for the former purpose (Björndahl et al., 2010), the latter concept raises an intriguing physiological question as to whether a high sperm survival after 24 h incubation is "good" or "bad". This is because the prolonged incubation of human spermatozoa under capacitating conditions leads to a high level of spontaneous acrosome reactions due to the lability of capacitated spermatozoa – but then having undergone the acrosome reaction the spermatozoa die (Mortimer et al., 1989). Consequently, a high proportion of dead spermatozoa after 24 h in a medium optimized for sperm capacitation could be seen as revealing a high physiological response of the man's spermatozoa rather than "toxicity", whereas in a sub-optimal capacitating medium a high level of sperm survival would not reflect a "good" result. It should also be noted that *in vivo* stored spermatozoa lose their capacity to fertilize long before motility decreases: "aged" but still motile spermatozoa lead to lower fertilization rates and cause chromosomal anomalies in embryos and hence an increased frequency of preimplantation losses (Martin-DeLeon et al., 1973; Tesh and Glover, 1979; Martin-DeLeon and Boice, 1982).

In any sperm motility bioassay for toxicity there should not only be an untreated control but also a known negative control, i.e. an aliquot of the sperm suspension should be exposed to a known toxic substance to control for the relative "hardiness" of the test sperm population.

Finally, it is essential that the method whereby the analyte sperm population is prepared must not expose the spermatozoa to any adverse factors that could themselves affect the sperm motility or survival Of particular importance is using a known safe sperm preparation method if using a washed sperm population as the analyte, i.e. either direct swim-up from semen or discontinuous density gradient centrifugation (Mortimer D, 2000; Björndahl et al., 2010). While the former method selects spermatozoa based on their motility, the latter selects the most mature spermatozoa – and arguments can be made for either

as the preferred technique for spermotoxicity bioassay applications, but either way the essential internal controls of the assay will ensure objective testing (e.g. Agarwal et al., 2008).

Human sperm vitality: These assays depend primarily on the quantification of the "live" sub-population using a dye exclusion technique, e.g. eosin-nigrosin or Hoechst 33258, but are more restricted to testing for sperm iso- and auto-immunity, or as extensions to assessments of sperm function (Mortimer et al., 1990; Mortimer, 1994; ESHRE Andrology Special Interest Group, 1996; Björndahl et al., 2003).

Human sperm morphology

Assessment of sperm morphology has been used extensively in studies on reproductive toxicology, both using the mouse model (Wyrobek et al., 1983a) and human spermatozoa (Wyrobek et al., 1983b). Human sperm morphology has also been used successfully in epidemiological studies on fertility (e.g. Jouannet et al., 1988; Slama et al., 2002; Attaman et al., 2012).

Of the several assessment schemes in current clinical use, those based on strict application of classification criteria and the inclusion of an index of sperm abnormality as an additional dimension of information to complement the (typically very low) % "normal" forms are likely to provide the most useful information (Mortimer and Menkveld, 2001; Auger, 2010). It is also recommended that the term "normal" be replaced by either "ideal" or "typical". The most common schemes for classifying human sperm morphology are based on either the Tygerberg Strict Criteria (Kruger et al., 1986; Menkveld et al., 1990) or the David system in France (David et al., 1975), although some centres still use the older WHO'92 scheme (World Health Organization, 1992). When assessments of sperm "normality" are made of the basis of strictness, there is no biologically significant difference between the WHO'92 and Tygerberg schemes (Mortimer and Menkveld, 2001), and this is the basis for the current scheme employed by the ESHRE Special Interest Group in Andrology (SIG-A: Björndahl et al., 2010; Barratt et al., 2011), which is similar to, but more informative than, the current WHO scheme (World Health Organization, 2010).

For the additional information on the degree of malformation of the abnormal spermatozoa, which is a negative predictor of biological function, the ESHRE Special Interest Group in Andrology scheme (Björndahl et al., 2010) uses the "teratozoospermia index" (TZI) and the French David scheme (Eustache and Auger, 2003) uses the "multiple anomalies index" (MAI). The TZI is average number of defective regions per abnormal spermatozoon: each abnormal cell having from 1 to 4 defects, assessed in the head, neck/midpiece and trail regions plus the presence of a cytoplasmic droplet.

As in all aspects of semen analysis, thorough training and quality control are essential to achieve robust standardization within and between laboratories (Auger et al., 2000; Franken et al., 2000; Björndahl et al., 2002; Eustache and Auger, 2003).

Human sperm function tests

There are numerous bioassays for evaluating different aspects of human sperm function *in vitro* that can be applied in certain situations for evaluating spermotoxicity under various conditions. The prime consideration when contemplating using such assays for this purpose is ensuring the physiological relevance of the endpoint in regard to the likely or potential circumstances under which exposure to the toxicant might occur. Ensuring physiological appropriateness of the manipulations, the circumstances, concentrations and durations of exposure, and the post-exposure incubation conditions and duration are all vital to designing a valid spermotoxicity sperm function bioassay. The omission or failure to achieve any one of these critical features could render a study meaningless – and hence allow a perceived "safe" substance or product into use with subsequent adverse outcomes and possible liability.

Sperm-mucus penetration: The first obstacle that human spermatozoa must face *in vivo* is the cervical mucus, into which they must migrate from the ejaculate. Because only the oestrogenic mucus secreted during the periovulatory period is receptive to penetration by spermatozoa, receptive cervical mucus is in very limited supply, with obvious opportunities for major biological variability between samples. As a consequence, various researchers have attempted to use either animal (e.g. bovine) mucus (Schütte, 1987; Barratt et al., 1992) or synthetic materials that mimic cervical mucus in their penetrability by human spermatozoa such as hyaluronic acid (Mortimer et al., 1990) or methylcellulose (Ivic et al., 2002). Although no objectively standardized protocols exist for currently available materials or products (Björndahl et al., 2010), such a bioassay would be highly advantageous.

Sperm hyperactivation: Although hyperactivation is a flagellar phenomenon, the only practical means for identifying spermatozoa showing this pattern of motility in a capacitating population requires specialized CASA techniques (ESHRE Andrology Special Interest Group, 1996). If all appropriate assay conditions are met, including careful separation of the spermatozoa from seminal plasma, specific physiological requirements for the incubation medium, and an adequate analysis preparation depth so as not to constrain flagellar movement, reliable analysis of human sperm hyperactivation is possible (ESHRE Andrology Special Interest Group, 1998; Mortimer ST, 2000), and can be used as the basis for a bioassay. However, the expression of spontaneous sperm hyperactivation is highly variable over time, requiring multiple time point assessments. Incorporation of the agonists progesterone

and pentoxifylline has allowed the definition of a single time point bioassay, similar to the "ARIC" test for studying the human sperm acrosome reaction (Björndahl et al., 2010), although its usefulness in spermotoxicity testing remains to be ascertained.

Other markers of sperm capacitation: Various other markers of the capacitation process have been described over the years, including binding of the lectin Concanavalin A (Con A) to the sperm surface (Ahuja, 1985; Cross and Overstreet, 1987), changes in chlortetracycline (CTC) fluorescence as a reflection of intracellular calcium levels (Lee et al., 1987), and the activation of phosphokinase A (PKA) and concomitant phosphorylation of tyrosine residues within the structures of the sperm tail (Moseley et al., 2005). Neither Con A binding nor CTC fluorescence remain in common usage, but the biochemical assays of PKA activation and tyrosine phosphorylation would seem amenable to use in studying the effect of potential or known spermotoxic substances on aspects of the sperm capacitation process.

Acrosome reaction: Because the fertilizing spermatozoon undergoes its acrosome reaction (AR) on the zona pellucida (Tesarik, 1989; Yanagimachi, 1994, 2011), the occurrence of spontaneous ARs during *in vitro* incubation is actually related to sperm pathology rather than to sperm physiology. Assessments of AR ability have focussed on two areas of sperm pathophysiology: (a) AR prematurity; and (b) AR insufficiency (ESHRE Andrology Special Interest Group, 1996). The former identifies an instability in a man's spermatozoa once they achieve the capacitated state, and is related to subfertility, compromised sperm-zona binding (Liu and Baker, 1994) and poor *in vitro* fertilization performance, while the latter signifies an impaired ability of a man's spermatozoa either to capacitate (fully) or for his capacitated spermatozoa to respond to the normal physiological trigger event and/or undergo the AR (Tesarik, 1989). Approximately 5% of infertility patients have an AR problem, about half AR insufficiency and half AR prematurity; intriguingly, both pathologies can occur in the same man (ESHRE Andrology Special Interest Group, 1996). From the fertility diagnostic perspective, comprehensive AR assessment for a patient involves assessing both the levels of spontaneous ARs under optimized capacitating conditions, and the occurrence of ARs following exposure to an appropriate agonist. In the absence of solubilized human zonae pellucidae, or biologically active rhZP3, the calcium ionophore A23187 remains the standard agonist within the format of an "ARIC" test (AR following ionophore challenge; Cummins et al., 1991), a standardized protocol for which has been established (ESHRE Andrology Special Interest Group, 1996; Björndahl et al., 2010).

Sperm-zona pellucida ("ZP") binding: After penetrating the cumulus oophorus, human spermatozoa bind tightly to the zona pellucida which induces a signal transduction cascade within the spermatozoon leading to the acrosome reaction. Two types of sperm-ZP binding tests have been described: (a) the original hemizona assay, exposing one half of a bisected oocyte to the test sperm population and the other half as the internal control (Burkman et al., 1988; Franken et al., 1989); and (b) a competitive binding test using intact ZPs in conjunction with mixtures of test and control sperm populations with different fluorescent labels (Liu et al., 1988). Tight binding of the spermatozoa to the ZP is the primary endpoint of these tests, which have a high predictive value for IVF success and a good ability to identify men at risk for failed or poor IVF fertilization (ESHRE Andrology Special Interest Group, 1996). The extremely limited availability of human ZPs precludes any routine use of these tests.

Zona-free hamster egg penetration test: Popular during the 1980s and early 1990s, the "hamster egg penetration test" (HEPT), or the "sperm penetration assay" (SPA) in the US literature, is now rarely used as a clinical test. While the value of the test in assessing sperm function remains unquestioned, it is technically complex and there are no widely-accepted, standardized protocols for its application (ESHRE Andrology Special Interest Group, 1996; Oehninger et al., 2000; Björndahl et al., 2010). Furthermore, in some jurisdictions the mixing of human and animal gametes is now prohibited by law.

Human IVF: While clearly not amenable for experimental applications, clinical human IVF might be seen as a valid trial study for certain clinical products that have evolved from established routine clinical practices. However, this should be preceded by studies that have not identified any evidence for adverse effects upon sperm physiology or functional potential, including IVF in a suitable animal model.

Human sperm DNA testing

Many types of test for sperm DNA damage or DNA integrity have been described and are discussed elsewhere (Barratt et al., 2010; Björndahl et al., 2010; Anton and Krawetz, 2012). It should be noted that those tests which measure real DNA damage will be better predictors of pregnancy than tests which measure only the "potential" for DNA damage, with the latter tests generally having lower predictive power. Because >90% of human DNA consists of non-protein-coding regions (introns) the probability of random DNA damage affecting protein-coding regions (exons) is very low, and consequently many spermatozoa bearing some DNA damage can produce viable pregnancies. Although the Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) test is often used to determine apoptosis in cells, it can also be used to detect sperm DNA fragmentation. Based on this, and the commercial availability of a test kit, it is currently recommended for measuring sperm DNA fragmentation in routine laboratory use, and a standardized protocol has been proposed (Björndahl et al., 2010). Analysis of its results by flow cytometry is recommended, but methods using fluorescence and light microscopy assessment are also available. Other recently described diagnostic tests of sperm DNA damage include the DNA breakage detection-fluorescence in situ hybridization assay ("DBD-FISH":

Fernández et al., 2011), the two-tailed Comet assay (Enciso et al., 2009), and the alkaline Comet assay – for which some clinical interpretation criteria exist (Simon et al., 2011). A modified comet assay "ReProComet", based on the addition of a protein extract from HeLa cells to agarose-embedded bovine spermatozoa on microscopic slides so that DNA breaks produced during adduct repair can be detected, has been reported for germ cell genotoxicity testing (Cordelli et al., 2007).

In terms of clinical predictive value the sperm chromatin structure assay (SCSA) has the most clinical data, and validated clinical interpretation guidelines are available: a low DNA Fragmentation Index (DFI <30%) being predictive for both natural fertility and pregnancy following IUI (Evenson et al., 2008). Since the SCSA is a "susceptibility" assay its results can be influenced by, for example, manipulating sperm zinc content. Because the DFI values used in the establishment of the interpretation criteria for the SCSA were derived using proprietary software to analyze the cytograms (SCSA Diagnostics Inc, Volga, SD, USA), only results obtained from a laboratory licensed to use this software should be interpreted using these guidelines.

Non-human sperm bioassays

Sperm motility: Multiple straws of cryopreserved bovine spermatozoa from an ejaculate, or pool of ejaculates, or fresh ejaculates that have either a large volume or contain very large numbers of spermatozoa, can be used for dose-response and/or multi-replicate studies (Petzoldt et al., 1985; Seibert et al., 1989). Cryopreserved material also permits repeat testing over time. Porcine sperm have also been used in this way (Andersson et al., 1998), and at least in regard to cereulide toxin, a cause of food poisoning, were found to be 100 times more sensitive than bovine spermatozoa (Jääskeläinen et al., 2003). While such studies might be used as sensitive screening tools, given inter-species differences they cannot entirely replace testing with human spermatozoa.

Sperm function tests: Similarly, tests of sperm function using material from domesticated or laboratory species could be used as screening assays, although there are few robust, established assays for non-human Eutherian spermatozoa, and laboratories performing such tests on a routine basis are relatively scarce.

In vitro fertilization: Integrated bioassays of sperm function based on animal IVF, e.g. bovine, represent a feasible, practical approach to screening for sperm toxicity (Lazzari et al., 2008) and could constitute a practical surrogate system since human IVF cannot be used as a general bioassay on ethical and moral grounds. Because of the ease with which mouse spermatozoa capacitate in vitro (Fraser, 1983), and because mouse embryos are less sensitive to bacterial endotoxins than human embryos (Randall and Gantt, 1990), this species is not well-suited for such testing. Given the possibility of inter-species differences, such a system still could not entirely replace testing with human spermatozoa, e.g. *in vitro* tests of sperm fertilizing ability.

Mouse embryo assay (MEA): The mouse embryo assay (MEA) is a common bioassay for toxicity and functionality testing of ART culture media or any device coming into contact with gametes (Gardner et al., 2005; Punt-van der Zalm et al., 2009), and is required by various professional standards (e.g. ASRM and SART, 2008) regulatory authorities (e.g. US FDA) on a per batch, pre-release basis. While the MEA is often more sensitive than a human sperm motility assay (Hughes et al., 2010), a negative MEA result does not guarantee that a product or device might not have some form of toxicity to spermatozoa, and hence andrology media and products are typically also tested using a sperm motility / survival assay.

Testing for sperm toxicity

International standardized protocols already exist for investigating male reproductive toxicity using animal models, e.g. OECD 1- or 2-generation reproductive toxicology studies, and should be applied and strictly complied with when performing such testing (see "Regulatory aspects of reproductive toxicology", above). Well-defined principles for designing studies on human male reproductive health (e.g. Buck Louis et al., 2008, Sharpe, 2009, 2010; Balabanič et al., 2011; Skakkebaek et al., 2011), and general human fertility in terms of time-to-pregnancy (e.g. Tingen et al., 2004), also exist. But, while many studies on human semen characteristics as indirect measures of fertility potential have been published, and significant advances have been made in ensuring methodological and technical standardization (e.g. Schrader et al., 1992; Brazil et al., 2004a,b), debate continues concerning difficulties of study design and subject recruitment to avoid possible population bias (e.g. Cohn et al., 2002; Eustache et al., 2004). It is hoped that recently proposed guidelines for the appraisal of studies based on semen quality endpoints will help improve the quality of research in this area (Sánchez-Pozo et al., 2012).

However, studies on direct sperm toxicity, typically using in vitro assessments of sperm fertilizing ability or DNA integrity, are uncommon and often difficult to interpret due to varied methodologies and technical differences (Anton and Krawetz, 2012; Sánchez-Pozo et al., 2012). Although it is hoped that genomic approaches will prove effective tools for studying genotoxicity in the future (Roy Choudhury et al., 2010;Kosova et al., 2012; Aston and Conrad, 2013), direct investigations on sperm (dys)function will likely remain important for establishing a lack of actual deleterious effect on sperm function. Consequently, we conclude this review with suggested guiding principles for developing not only biologically appropriate strategies for evaluating possible sperm toxicity (Fig. 4), but also minimum standards for *in vitro* bioassays of human sperm functional potential (requiring technical

standardization, objective analysis, operator competence, and valid statistical analysis and interpretation of the results; see Sánchez-Pozo et al., 2012). Ideally, test protocols should be based on reference analytical methods that have been approved by Expert Groups, convened by either a governmental regulatory agency (e.g. the US EPA) or an international professional society. In summary:

- Bioassays of sperm pathophysiology must be developed and validated by scientists experienced in spermatology or laboratory andrology to ensure not only their physiological and methodological correctness, but also that endpoints are defined and identified objectively and unambiguously.
- Studies must be designed critically, so as to reliably identify an adverse effect – or the lack thereof – and not just to show "equivalence" by failing to reach statistical significance due to inherent variability of the data, e.g. by basing the assay endpoint on too small a number of spermatozoa, or inadequate replicates.
- A standardized method should be designed to minimize inherent errors, and the uncertainty of measurement of the endpoint of interest defined; these are critical for intra- and inter-assay reproducibility. This includes counting sufficient spermatozoa from replicate preparations made from replicate treatments of each subject / concentration / dosage / time point permutation in order to minimize sampling and counting errors. Wherever practically possible all assessments must be made "blind", i.e. with the observer unaware of the provenance of each slide being read, to remove the risk of observer bias – both intentional and unintentional. This is especially important when relying on visual, subjective appraisal of the endpoint.

Appropriate degree and duration of exposure to test substance: It is essential that the manner of exposure of the analyte spermatozoa to the test substance is appropriate; it needs to reflect physiology, the manner in which spermatozoa might be exposed to the substance in the real world – including dosage and duration of exposure – and the environment in which that exposure occurs. For example, the initial evaluation of a potential vaginal lubricant product must expose spermatozoa still in seminal plasma to the actual product at a concentration similar to which they might experience – or a high as they might possibly be exposed – within the vagina during intercourse *in vivo*, e.g. 10% (v/v) for perhaps 30 minutes (e.g. Agarwal et al., 2008). Testing in a sperm motility assay could well be the first step, but effective penetration from semen into cervical mucus post-exposure is a logical second stage, followed by testing for possible effects on sperm function ideally using spermatozoa prepared from semen post-exposure.

However, when screening chemical compounds in isolation it will be difficult, if not impossible, to consider all possible synergistic and additive effects that might arise in future mixtures containing the substance. Therefore a final product must be tested directly for spermotoxic effects, rather than relying on previous absences of effect in tests on its constituent materials.

Controls, test subjects / sperm donors, and replicates: Especially when working with a biological material such as spermatozoa as the analyte, it is essential that both positive and negative controls are included in all tests control for sperm quality and to identify non-specific effects. Sperm functional competence varies markedly between men, ranging from "super stud" sperm donors to men who are functionally sterile, and consequently epidemiological studies on male fertility must pay close attention to avoiding subject recruitment bias (Cohn et al., 2002; Eustache et al., 2004; Sánchez-Pozo et al., 2012). Similarly, a laboratory performing in vitro tests of sperm function must also avoid inappropriate bias in the provenance of the sperm samples from which the analyte spermatozoa are being derived, e.g. a population of selected research donors who might be drawn from a population of "retired" therapeutic sperm donors). Compared to such men's spermatozoa, those from men more representative of the general population might show a greater sensitivity to a toxicant, and spermatozoa from subfertile men could be even more sensitive – and perhaps therefore more appropriate as test subjects. Because variable duration of exposure to seminal plasma influences both the functional properties and DNA vulnerability of spermatozoa, and because routines obviously vary between different laboratories, it is likely that the unpredictable extent and variability of such exposure in diagnostic laboratories could preclude the use of diagnostic specimens in controlled scientific studies and validations in many laboratories.

Therefore, rather than simply specifying a minimum number of subjects, it is essential that a spectrum of donors (e.g. selected research donors and general volunteers) as well as patients (male partners of couples attending an infertility clinic) be included to ensure broad-based investigation of a potential toxicant against a typical population that might be exposed to it. Combined with the possible magnitude of possible biological effect(s) this complicates establishing simple generic rules in advance, although a reasonable test population to support a claim that a substance or product does not show spermotoxicity might be 20 men, at least half of whom are in the "at-risk" infertility clinic or at least in the "trying-to-conceive" category. For a product to which spermatozoa will specifically be exposed during the normal course of its use (e.g. a vaginal lubricant) then this number should be considered a definite minimum.

When setting scientific limits between a "toxic effect" and "no toxic effect" a general risk assessment of the nature of the actual effect must be included. For instance, a situation where a transgenerational effect might be induced is far more serious than if no individual would be harmed – such as if exposed spermatozoa were simply unable to fertilize. The first situation would necessitate extensive assessments in order to decrease the influence of random variation, while in the latter case cost-benefit

and risk analysis would weigh the risk for sperm dysfunction against the possible benefits of the exposure.

Operator training and competence: Because sperm-based bioassays typically rely on visual appraisal for the assessment of results, it is essential that – just like reliable diagnostic semen analyses – they are only performed by properly trained, experienced laboratory andrology staff (MacLeod & Gold, 1951; Eliasson, 1977; Mortimer, 1994; Björndahl et al., 2010; Barratt et al., 2011; Sánchez-Pozo et al., 2012). In order to achieve the necessary levels of accuracy and precision, reflected in a suitably low uncertainty of measurement (e.g. ±10%; Björndahl et al., 2010), operator training must be based on objectively defined assessment criteria and objective definitions of competency. These must be followed by regular internal quality control (IQC) exercises, and adequate performance in an external quality assurance (EQA) programme that incorporates an effective quality improvement capability (Björndahl et al., 2010; Sánchez-Pozo et al., 2012).

Statistical analysis and results reporting: Given the wide range of possible effects on sperm function, as well as likely inter-individual variability in their magnitude and analytical uncertainty of measurement, careful power calculations will be required to establish appropriate subject and replicate numbers on a study by study basis. Rigorous statistical analysis of assay data must use methods that will not obscure differences, e.g. not trying to conceal or reduce variability by expressing results as mean±SEM (always use mean ±SD for suitable data), proper use of parametric and non-parametric testing, use of paired tests when valid, and avoiding the use of simplistic correlations when making comparisons (see Chapter 10 of Björndahl et al., 2010). Results must be interpreted appropriately: testing must show clear a lack of adverse effect (or perhaps a beneficial effect) for a substance of product to be considered "sperm-safe"; and comments on perceived "trends" have no value, only results with statistical significance can be interpreted and reported.

A minimum panel of tests to establish a lack of in vitro sperm toxicity

A possible panel of "current generation" tests covering the spectrum of biological functions that spermatozoa must be able to perform or achieve without compromise following exposure to a substance in order for that substance to be described as "sperm safe" are shown in Table 4. Referenced methods are current established testing protocols for diagnostic purposes; more streamlined, automated analytical protocols will likely be required for high throughput screening purposes. In general, assessments lower in the Table are more complex and costly, and would most likely be preceded by tests listed higher in the Table. The composition of specific testing packages will be influenced by various practical factors such as a particular substance's expected mode of action and cost-effectiveness of available testing technology.

Conclusions and summary of recommendations

While the authors do not seek to dictate to industry or regulatory authorities, based on our review of the literature we believe that it is reasonable to expect sound evidence of safety, as summarized in Table 5 before claiming that a product is "sperm-safe". The need for such evidence is most certainly in the public interest, especially that of couples who are trying to conceive. It is hoped that international andrology societies or special interest groups will embrace our general principles and promote the development of specific consensus protocols for evaluating *in vitro* sperm toxicity, similar to those already developed for *in vivo* animal bioassays for reproductive toxicity, along with standard operating procedures for specific testing methods.

Note: In the following recommendations, "substance" includes individual chemicals, mixtures of chemicals, pharmaceuticals or nutraceuticals, as well as compounded products such a creams or lubricants.

1) A claim that a substance is "sperm-safe" can only be made subsequent to the performance of objective, properly designed and carried out experimental studies using appropriate bioassays for all pertinent aspects of sperm production (including neuroendocrine and testicular aspects), spermotoxicity and biocompatibility (especially for substances with topical or intimate application), not just on prototype formulations but also on final production formulations and revised or "improved" formulations. Such studies must include an adequately representative population of subjects, especially when couples who are trying to conceive will be exposed.

   As a corollary to this, any claim of "sperm-safe" should be specified in terms of those aspects of sperm function – or biological endpoints – that are NOT affected by the substance, e.g. male endocrine function, sperm formation (number, morphology), sperm motility, sperm DNA vulnerability, fertilization, embryo development, pregnancy, delivery rate, birth of a healthy child, etc. A statement of "sperm-safe" for a substance should indicate that all possible endpoints have been evaluated and that there is adequate evidence that they are not affected. A lack of proof for an adverse effect cannot be taken as evidence for there being no effect.

2) Extrapolation that a substance is "sperm-safe" from a predicate substance must be done only under the strictest of circumstances, even for a revised or "improved" formulation.

3) Presumption that a substance is not toxic to spermatozoa cannot be based on either:

a) it only comprising constituents that are generally regarded as safe (GRAS) by regulatory authorities for other purposes;
b) none of its constituents being currently listed elsewhere as spermotoxic; or
c) assumptions that components have been used for different purposes in other products and must ergo be safe (e.g. glycerol as a cryoprotectant *c.f.* its use in a lubricant).

4) Appropriate bioassay verification of lack of sperm toxicity must be integral to the QC testing for individual production batches of a substance that will, or even might, come into contact with spermatozoa through its expected use. These must be in addition to standard QC tests for lack of endotoxin contamination and sterility.

5) Bioassays for in vitro sperm toxicity must be based on appropriate physiology, be technically robust, achieve adequate levels of uncertainty of measurement that allow confident interpretation of assay results, be performed by properly trained and qualified laboratory personnel, and be interpreted by someone with expert knowledge of sperm physiology and/or andrology. See "A minimum panel of tests to establish a lack of in vitro sperm toxicity", above.

6) A suitable authority or organization* should maintain registries of:
   a) products that have been established, using validated methods, as being "sperm-safe"; and
   b) substances that have been established as having one or more activities that are toxic to sperm production or sperm function.

   Substances or products not listed must be considered suspect until confirmed to not have any toxic effects on sperm production or sperm physiology by appropriate testing.

   *e.g. A working group or committee of the ESHRE Andrology SIG or the International Society for Andrology.

7) The limited data available on human subjects, and in certain instances inconsistent data across studies, highlight the need for further epidemiological and reproductive toxicological research on the different classes of EDCs which will contribute to our overall knowledge on "sperm-safe" compounds.

8) The relationship between EDC exposure and male reproductive and developmental health includes the changes in exposure levels among populations over time. The effects of complex environmental EDC mixtures on spermatogenesis, as well as the trans-generational effects on the offspring of exposed males, including their sperm function, should receive more attention.

9) Innovations including improved biomarkers of exposure and more sophisticated statistical methods that deal with multiple exposures simultaneously should be developed and implemented.

Authors' roles

All authors contributed substantively to the scope, writing and editing of the manuscript; DM acted as coordinating author, other co-authors are listed alphabetically.

Funding

No research funding was received by the authors in regard to this manuscript. Publication of this Open Access Supplement was supported financially by INGfertility, who had no input into the writing of the manuscript.

Conflict of interest

None of the authors have received any funding or other payments or gifts that have had any influence on the content of this manuscript. DM is employed full-time by Oozoa Biomedical which receives revenues from a wide range of commercial and clinical clients that varies over time.

Acknowledgements

The authors would like to express their gratitude to the reviewers of the original manuscript for their perceptive, supportive and constructive criticisms, and for their suggestions which contributed significantly to the quality of the final article.

Bibliography

Agarwal A, Deepinder F, Cocuzza M, Short RA, Evenson DP. Effect of vaginal lubricants on sperm motility and chromatin integrity: a prospective comparative study. *Fertil Steril* 2008: **89**; 375-379.

Agarwal A, Deepinder F, Sharma RK, Ranga G, Li J. Effect of cell phone usage on semen analysis in men attending infertility clinic: an observational study. *Fertil Steril* 2008: **89**; 124-128.

Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol* 2008: **59**; 2-11.

Ahuja K. Carbohydrate determinants involved in mammalian fertilization. *Am J Anat* 1985: **174**; 207-223.

Aitken RJ, Baker MA, De Iuliis GN, Nixon B. New insights into sperm physiology and pathology. *Handbook Exp Pharmacol* 2010: **198**; 99-115.

Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1987: **81**; 459-469.

Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod* 2010: **16**; 3-13.

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998: **59**; 1037-1046.

Aitken RJ, Koopman P, Lewis SEM. Seeds of concern. *Nature* 2004: **432**; 48-52.

Aitken RJ, McLaughlin EA. Molecular mechanisms of sperm capacitation: progesterone-induced secondary calcium oscillations reflect the attainment of a capacitated state. *Soc Reprod Fertil Suppl* 2007: **63;** 273-293.

Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. *Mol Hum Reprod* 2007: **13;** 203-211.

Ajduk A, Malagocki A, Maleszewski M. Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca^{2+} oscillations. *Reprod Biol* 2008: **8;** 3-22.

Akre O, Cnattingius S, Bergström R, Kvist U, Trichopoulos D, Ekbom A. Human fertility does not decline: evidence from Sweden. *Fertil Steril* 1999: **71**; 1066-1069.

Alexander BH, Checkoway H, van Netten C, Muller CH, Ewers TG, Kaufman JD, Mueller BA, Vaughan TL, Faustman EM. Semen quality of men employed at a lead smelter. *Occup Environ Med* 1996: **53**; 411-416.

Alvarez JG, Storey BT. Spontaneous lipid peroxidation in rabbit epididymal spermatozoa. *Biol Reprod* 1982: **27**; 1102-1108.

Alvarez JG, Storey BT. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev* 1995: **42**; 334-346.

Amann RP, Howards SS. Daily spermatozoal production and epididymal spermatozoal reserves of the human male. *J Urol* 1980: **124**; 211-215.

Amory JK, Bremner WJ. The use of testosterone as a male contraceptive. *Baillieres Clin Endocrinol Metab* 1998: **12**; 471-484.

Amory JK, Page ST, Bremner WJ. Drug insight: Recent advances in male hormonal contraception. *Nat Clin Pract Endocrinol Metab* 2006: **2**; 32-41.

Amory JK, Wang C, Swerdloff RS, Anawalt BD, Matsumoto AM, Bremner WJ, Walker SE, Haberer LJ, Clark RV. The effect of 5α-reductase inhibition with dutasteride and finasteride on semen parameters and serum hormones in healthy men. *J Clin Endocrinol Metab* 2007: **92**;1659-1665.

Anderson L, Lewis SE, McClure N. The effects of coital lubricants on sperm motility in vitro. *Hum Reprod* 1998: **13**; 3351-3356.

Andersson MA, Mikkola R, Kroppenstedt RM, Rainey FA, Peltola J, Helin J, Sivonen K, Salkinoja-Salonen MS.The mitochondrial toxin produced by Streptomyces griseus strains isolated from an indoor environment is valinomycin. *Appl Environ Microbiol* 1998: **64;** 4767-4773.

Anton E, Krawetz SA. Spermatozoa as biomarkers for the assessment of human male infertility and genotoxicity. *Syst Biol Reprod Med* 2012: **58;** 41-50.

Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* 2005: **308**; 1466-1469.

Anway MD, Memon MA, Uzumcu M, Skinner MK. Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *J Androl* 2006: **27**; 868-879.

Appell RA, Evans PR. The effect of temperature on sperm motility and viability. *Fertil Steril* 1977: **28**; 1329-1332.

ASRM and SART. Revised guidelines for human embryology and andrology laboratories. *Fertil Steril* 2008: **90;** S45-S59.

Aston KI, Conrad DF. A review of genome-wide approaches to study the genetic basis for spermatogenic defects. In Carrell DT and Aston KI (eds) Spermatogenesis: Methods and Protocols. *Methods in Molecular Biology* 2013: **927;** 397-410.

ATDSR. Toxicological Profile for Di-(2-ethylhexyl) phthalate (DEHP). 2002. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta.

Attaman JA, Toth TL, Furtado J, Campos H, Hauser R, Chavarro JE. Dietary fat and semen quality among men attending a fertility clinic. *Hum Reprod* 2012: **27;** 1466-1474.

Auger J. Assessing human sperm morphology: top models, underdogs or biometrics? *Asian J Androl* 2010: **12;** 36-46.

Auger J, Eustache F, Ducot B, Blandin T, Daudin M, Diaz I, El Matribi S, Gony B, Keskes L, Kolbezen M et al. Intra- and inter-individual variability in human sperm concentration, motility and vitality assessment during a workshop involving ten laboratories. *Hum Reprod* 2000: **15;** 2360-2368.

Austin CR. The capacitation of the mammalian sperm. *Nature* 1952: **170**; 326.

Avendaño C, Mata A, Sanchez Sarmiento CA, Doncel GF. Use of laptop computers connected to internet through Wi-Fi decreases human sperm motility and increases sperm DNA fragmentation. *Fertil Steril* 2012: **97**; 39-45.

Bailey JL. Factors regulating sperm capacitation. *Syst Biol Reprod Med* 2010: **56**; 334-348.

Balabanič D, Rupnik M, Klemenč ič AK. Negative impact of endocrine-disrupting compounds on human reproductive health. *Reprod Fertil Dev* 2011: **23;** 403-16.

Barratt CL, Aitken RJ, Björndahl L, Carrell DT, de Boer P, Kvist U, Lewis SE, Perreault SD, Perry MJ, Ramos L, Robaire B, Ward S, Zini A. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications – a position report. *Hum Reprod* 2010: **25**; 824-838.

Barratt CLR, Björndahl L, Menkveld R, Mortimer D. The ESHRE Special Interest Group for Andrology Basic Semen Analysis Course: A continued focus on accuracy, quality, efficiency and clinical relevance. *Hum Reprod* 2011: **26**; 3207-3212.

Barratt CL, McLeod ID, Dunphy BC, Cooke ID. Prognostic value of two putative sperm function tests: hypo-osmotic swelling and bovine sperm mucus penetration test (Penetrak). *Hum Reprod* 1992: **7**; 1240-1244.

Barroso G, Valdespin C, Vega E, Kershenovich R, Avila R, Avendaño C, Oehninger S. Developmental sperm contributions: fertilization and beyond. *Fertil Steril* 2009: **92;** 835-848.

Bars R, Fegert I, Gross M, Lewis D, Weltje L, Weyers A, Wheeler JR, Galay-Burgos M. Risk assessment of endocrine active chemicals: identifying chemicals of regulatory concern. *Regul Toxicol Pharmacol* 2012: **64;** 143-154.

Baska KM, Manandhar G, Feng D, Agca Y, Tengowski MW, Sutovsky M, Yi YJ, Sutovsky P. Mechanism of extracellular ubiquitination in the mammalian epididymis. *J Cell Physiol* 2008: **215**; 684-696.

Bedford JM. The status and the state of the human epididymis. *Hum Reprod* 1994: **9;** 2187-2199.

Bedford JM. Enigmas of mammalian gamete form and function. *Biol Rev* 2004: **79**; 429-460.

Begay O, Jean-Pierre N, Abraham CJ, Chudolij A, Seidor S, Rodriguez A, Ford BE, Henderson M, Katz D, Zydowsky T, Robbiani M, Fernández-Romero JA. Identification of personal lubricants that can cause rectal epithelial cell damage and enhance HIV Type 1 replication *in vitro*. *AIDS Res Hum Retroviruses* 2011: **27**; 1019-1024.

Beischlag TV, Luis MJ, Hollingshead BD, Perdew GH. The aryl hydrocarbon receptor complex and the control of gene expression. *Crit Rev Euraryot Gene Expr* 2008: **18;** 207-250.

Benoff S, Cooper GW, Hurley I, Mandel FS, Rosenfeld DL, Scholl GM, Gilbert BR, Hershlag A. The effect of calcium ion channel blockers on sperm fertilization potential. *Fertil Steril* 1994: **62**; 606-617.

Benoff S, Jacob A, Hurley IR. Male infertility and environmental exposure to lead and cadmium. *Hum Reprod Update* 2000: **6;** 107-121.

Berkovitz A, Eltes F, Ellenbogen A, Peer S, Feldberg D, Bartoov B. Does the presence of nuclear vacuoles in human sperm selected for ICSI affect pregnancy outcome? *Hum Reprod* 2006: **21;** 1787-1790.

Berndtson EB. Comparative reliability and sensitivity of different methods for assessing treatment effects on sperm production. *Anim Reprod Sci* 2008: **105**; 5-22.

BfR (Bundesinstitut für Risikobewertung). Final joint DE-UK position. Regulatory definition of an endocrine disrupter in relation to potential threat to human health. 2011: available at www.bfr.bund.de accessed 22 October 2012.

Björndahl L, Kvist U. Importance of zinc for human sperm head-tail connection. *Acta Physiol Scand* 1982: **116**; 51-55.

Björndahl L, Kvist U. Loss of an intrinsic capacity for human sperm chromatin decondensation. *Acta Physiol Scand* 1985: **124**; 189-194.

Björndahl L, Kvist U. Human sperm chromatin stabilization: a proposed model including zinc bridges. *Mol Hum Reprod* 2010: **16**; 23-29.

Björndahl L, Barratt CL, Fraser LR, Kvist U, Mortimer D. ESHRE basic semen analysis courses 1995-1999: immediate beneficial effects of standardized training. *Hum Reprod* 2002: **17;** 1299-1305.

Björndahl L, Söderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum Reprod* 2003: **18**; 813-816.

Björndahl L, Mortimer D, Barratt CLR, Castilla JA, Menkveld R, Kvist U, Alvarez JG, Haugen TB. A Practical Guide to Basic Laboratory Andrology. 2010. Cambridge University Press, Cambridge,UK.

Boitrelle F, Ferfouri F, Petit JM, Segretain D, Tourain C, Bergere M, Bailly M, Vialard F, Albert M, Selva J. Large human sperm vacuoles observed in motile spermatozoa under high magnification: nuclear thumbprints linked to failure of chromatin condensation. *Hum Reprod* 2011: **26;** 1650-1658.

Bonde JP. Male reproductive organs are at risk from environmental hazards. *Asian J Androl* 2010: **12**; 152-156.

Bornman MS, Barnhoorn IEJ, de Jager C, Veeramachaneni DNR. Testicular microlithiasis and neoplastic lesions in wild eland (Tragelaphus oryx): possible effects of exposure to environmental pollutants? *Environ Res* 2010: **110**; 327-333.

Bornman MS, de Jager C, Vorster H, Burger AEC, Apps PJA. p-Nonylphenol in food packaging material: An absorption study. Congress on Current Aspects of Reproductive Biology. 10th Biennial Meeting of SASRSS, August 1997, pp. 22-25.

Boscolo P, Sacchattoni-Longrocino G, Ranelletti FO, Gioia A, Carmignani M. Effects of long term cadmium exposure on the testes of rabbits: ultrastructural study. *Toxicol Lett* 1985: **24**; 145-149.

Botella-Llusia, J. Measurement of linear progression of the human spermatozoon as an index of male fertility. *Int J Fertil* 1956: **1;** 113-130.

Boyers SP, Corrales MD, Huszar G, DeCherney AH. The effects of Lubrin on sperm motility in vitro. *Fertil Steril* 1987: **47**; 882-884.

Bracken MB, Eskanazi B, Sachse K, McSharry J-E Hellenbrand K. Leo-Summers L. Association of cocaine use with sperm concentration, motility

morphology. *Fertil Steril* 1990: **53**; 315-322.

Branigan EF, Estes MA , Muller CH. Advanced semen analysis: A simple screening test to predict intrauterine insemination success. *Fertil Steril* 1999: **71;** 547-551.

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988: **332**; 459-461.

Braw-Tal, R. Endocrine disruptors and timing of human exposure. *Pediatr Endocrinol Rev* 2010: **8;** 41-46.

Brazil C, Swan SH, Drobnis EZ, Liu F, Wang C, Redmon JB, Overstreet JW; Study for Future Families Research Group. Standardized methods for semen evaluation in a multicenter research study. *J Androl* 2004a: **25;** 635-644.

Brazil C, Swan SH, Tollner CR, Treece C, Drobnis EZ, Wang C, Redmon JB, Overstreet JW; Study for Future Families Research Group. Quality control of laboratory methods for semen evaluation in a multicenter research study. *J Androl* 2004b: **25;** 645-656.

Brenker C, Goodwin N, Weyand I, Kashikar ND, Naruse M, Krähling M, Müller A, Kaupp UB, Strünker T. The CatSper channel: a polymodal chemosensor in human sperm. *EMBO J* 2012: **31;** 1654-1665.

Brindley GS. Deep scrotal temperature and the effect on it of clothing, air temperature, activity, posture and paraplegia. *Br J Urol* 1982: **54;** 49-55.

Buck GM, Lynch CD, Stanford JB, Sweeney AM, Schieve LA, Rockett JC, Selevan SG, Schrader SM. Prospective pregnancy study designs for assessing reproductive and developmental toxicants. *Environ Health Perspect* 2004: **112;** 79-86.

Buck Louis GM, Gray LE Jr, Marcus M, Ojeda SR, Pescovitz OH, Witchel SF, Sippell W, Abbottn DH, Soto A, Tyl RW et al. Environmental factors and puberty timing: expert panel research needs. *Pediatrics* 2008: **112;** 192-207.

Buffone MG, Ijiri TW, Cao W, Merdiushev T, Aghajanian HK, Gerton GL. Heads or tails? Structural events and molecular mechanisms that promote mammalian sperm acrosomal exocytosis and motility. *Mol Reprod Dev* 2012: **79;** 4-18.

Bujan L, Daudin M, Charlet JP, Thonneau P, Mieusset R. Increase in scrotal temperature in car drivers. *Hum Reprod* 2000: **15;** 1355-1357.

Burkman LJ, Coddington CC, Franken DR, Krugen TF, Rosenwaks Z, Hodgen GD. The hemizona assay (HZA): development of a diagnostic test for binding of human spermatozoa to the human zona pellucida to predict fertilization potential. *Fertil Steril* 1988: **49**; 688-693.

Calogero A, Polosa R, Perdichizzi A, Guarino F, La Vignera S, Scarfia A, Fratantonio E, Condorelli R, Bonanno O, Barone N et al. Cigarette smoke extract immobilizes human spermatozoa and induces sperm apoptosis. *Reprod Biomed Online* 2009: **19;** 564-571

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *BMJ* 1992: **305;** 609-613.

Carrell DT. Contributions of spermatozoa to embryogenesis: assays to evaluate their genetic and epigenetic fitness. *Reprod Biomed Online* 2008: **16**; 474-484.

Carrell DT, Hammoud SS. The human sperm epigenome and its potential role in embryonic development. *Mol Hum Reprod* 2010: **16;** 37-47.

Carrell DT, Liu L. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. *J Androl* 2001: **22**; 604-610.

Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermatogenesis: what is the link? *Hum Reprod Update* 2007: **13**; 313-327.

Casswall T H, Björndahl L, Kvist U. Cadmium interacts with the zinc-dependent stability of the human sperm chromatin. *J Trace Elem Electrolytes Health Dis* 1987: **1**; 85-87.

Castle L. Chemical migration into food: an overview. In Castle L (ed), Chemical Migration and Food Contact Materials. 2007. CRC Press/Woodehead Publishing Ltd, Cambridge,UK, pp. 1-13.

Cavallini G, Ferraretti AP, Gianaroli L, Biagiotti G, Vitali G. Cinnoxicam and L-carnitine/acetyl-L-carnitine treatment for idiopathic and varicocele-associated oligoasthenospermia. *J Androl* 2004: **25**; 761-770.

Centola GM, Cartie RJ, Cox C. Differential responses of human sperm to varying concentrations of pentoxyfylline with demonstration of toxicity. *J Androl* 1995: **16;** 136-142.

Ceretti E, Zani C, Zerbini I, Guzzella L, Scaglia M, Berna V, Donato F, Monarca S, Feretti D. Comparative assessment of genotoxicity of mineral water packed in polyethylene terephthalate (PET) and glass bottles. *Water Res* 2010: **44**; 1462-1470.

Chang H, Suarez SS. Rethinking the relationship between hyperactivation and chemotaxis in mammalian sperm. *Biol Reprod* 2010: **83**; 507-513.

Chang H, Suarez SS. Unexpected flagellar movement patterns and epithelial binding behavior of mouse sperm in the oviduct. *Biol Reprod* 2012: **86;** 140, 1-8.

Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* 1951: **168**; 697-698.

Chapin RE, Sloane RA, Haseman JK. The relationships among reproductive endpoints in Swiss mice, using the reproductive assessment by continuous breeding database. *Fundam Appl Toxicol* 1997: **38**; 129-142.

Cherry N, Moore H, McNamee R, Pacey A, Burgess G, Clyma JA, Dippnall M, Baillie H, Povey A. Occupation and male infertility: glycol ethers and other exposures. *Occup Environ Med* 2008: **65**; 708-714.

Chi HJ, Kim JH, Ryu CS, Lee JY, Park JS, Chung DY, Choi SY, Kim MH, Chun EK, Roh SI. Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. *Hum Reprod* 2008: **23**; 1023-1028.

Christensen BC, Marsit CJ. Epigenomics in environmental health. *Front Genet* 2011: **2;** 84.

Claassens OE, Wehr JB, Harrison KL. Optimizing sensitivity of the human sperm motility assay for embryo toxicity testing. *Hum Reprod* 2000: **15**;

1586-1591.

Claudio L, Bearer CF, Wallinga D. Assessment of the U.S. Environmental Protection Agency methods for identification of hazards to developing organisms. Part I: The reproduction and fertility testing guidelines. *Am J Ind Med* 1999: **35**; 543-553.

Clermont Y. Spermatogenesis in man. A study of the spermatogonial population. *Fertil Steril* 1966: **17;** 705-721.

Coccia ME, Becattini C, Criscuoli L, Fuzzi B, Scarselli G. A sperm survival test and in-vitro fertilization outcome in the presence of male factor infertility. *Hum Reprod* 1997: **12**; 1969-1973.

Coetzee K, Kruge TF, Lombard CJ. Predictive value of normal sperm morphology: a structured literature review. *Hum Reprod Update* 1998: **4;** 73-82.

Cohn BA, Overstreet JW, Fogel RJ, Brazil CK, Baird DD, Cirillo PM. Epidemiologic studies of human semen quality: considerations for study design. *Am J Epidemiol* 2002: **155;** 664-671.

Colborn T, Dumanoski D, Myers JP. Our Stolen Future: Are We Threatening Our Fertility, Intelligence, and Survival? – A Scientific Detective Story. 1997. Plume / Penguin Books USA, New York, USA.

See also http://www.ourstolenfuture.org/basics/chemlist.htm accessed 22 October 2012.

Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 1993: **101**; 378-384.

Collodel G, Scapigliati G, Moretti E. Spermatozoa and chronic treatment with finasteride: a TEM and FISH study. *Arch Androl* 2007: **53**;229-233.

Comhaire FH, Mahmoud A. The role of food supplements in the treatment of the infertile man. *Reprod Biomed Online* 2003: **7**; 385-391.

Cordelli E, Fresegna AM, D'Alessio A, Eleuteri P, Spanò M, Pacchierotti F, Villani P. ReProComet: a new in vitro method to assess DNA damage in mammalian sperm. *Toxicol Sci* 2007: **99;** 545-552.

Correa LM, Thomas A, Meyers SA. The macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. *Biol Reprod* 2007: **77**; 942-953.

Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J, Publicover S. Ca^{2+}-stores in sperm: their identities and functions. *Reproduction* 2009: **138;** 425-437.

Coutts SM, Fulton N, Anderson RA. Environmental toxicant-induced germ cell apoptosis in the human fetal testis. *Hum Reprod* 2007: **22;** 2912–2918.

Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD. Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. *Fertil Steril* 1988: **50;** 314-320.

Cross NL. Effect of methyl-beta-cyclodextrin on the acrosomal responsiveness of human sperm. *Mol Reprod Dev* 1999: **53**; 92-98.

Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. *Biol Reprod* 1988: **38**; 235-244.

Cross NL, Overstreet, JW. Glycoconjugates of the human sperm surface: distribution and alterations that accompany capacitation in vitro. *Gamete Res* 1987: **16**; 23-35.

Cummins JM, Pember SM, Jequier AM, Yovich JL, Hartmann PE. A test of the human sperm acrosome reaction following ionophore challenge. Relationship to fertility and other seminal parameters. *J Androl* 1991: **12**; 98-103.

Damstra T, Barlow S, Bergman A, Kavlock R, Van der Kraak G. Global Assessment of the State-of-the-Science of Endocrine Disruptors. 2002. WHO publication no. WHO/PCS/EDC/02.2. World Health Organization, Geneva, Switzerland.

Danish Environmental Protection Agency. Male reproductive health and environmental chemicals with estrogenic effects. Miljoproject 290. 1995. Ministry of Environment and Energy, Danish Environmental Protection Agency, Copenhagen, Denmark.

Dart RC (ed). Medical Toxicology 3rd edition. 2003. Lippincott Williams & Wilkins, Philadelphia, USA.

David G, Bisson JP, Czyglik F, Jouannet P, Gernigon C. Anomalies morphologiques du spermatozoïde humain. 1) Propositions pour un système de classification. *J Gyn Obstet Biol Reprod* 1975: **4 (suppl.1);** 17–36.

de Boer P, Ramos L, de Vries M, Gochhait S. Memoirs of an insult: sperm as a possible source of transgenerational epimutations and genetic instability. *Mol Hum Reprod* 2010: **16**; 48-56.

De Jager C, Bornman MS, van der Horst G. I. The effect of p-nonylphenol, an environmental toxicant with oestrogenic properties, on fertility potentila in adult male rats. *Andrologia* 1999a: **31**; 99-106.

De Jager C, Bornman MS, Oosthuizen JMC. II. The effect of p-nonylphenol on the fertility potential of male rats after gestational, lactational and direct exposure. *Andrologia* 1999b: **31**; 107-113.

De Jonge C. Biological basis for human capacitation. *Hum Reprod Update* 2005: **11**; 204-215.

De Jonge CJ, Centola GM, Reed ML, Shabanowitz RB, Simon SD, Quinn P. Human sperm survival assay as a bioassay for the assisted reproductive technologies laboratory. *J Androl* 2003: **24**; 16-18.

De Iuliis DN, Newey RJ, King BV, Aitken RJ. Mobile phone radiation indices reactive oxygen species production and DNA damage in human spermatozoa *in vitro*. *PLoS One* 2009: **4**; e6446.

de Kretser, DM. Ultrastructural features of human spermiogenesis. *Z Zellforsch Mikrosk Anat* 1969: **98**; 477-505.

de Lamirande E, Gagnon C. Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. *Fertil Steril* 1993: **59**; 1291-1295.

de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. *Rev Reprod* 1997: **2**; 48-54.

Dean J. The enigma of sperm-egg recognition in mice. *Soc Reprod Fertil Suppl* 2007: **63**; 359-365.

Delbès G, Hales BF, Robaire, B. Toxicants and human sperm chromatin integrity. *Mol Hum Reprod* 2010: **16**; 14-22.

Delbes G, Levacher C, Duquenne C, le Racine C, Pakarinen P, Habert R. Endogenous estrogens inhibit mouse fetal leydig cell development via estrogen receptor alpha. *Endocrinol* 2005: **146**; 2454-2461.

Deng F, Tao FB, Li DY, Xu YY, Hao JH, Sun Y. Effects of growth environments and two environmental endocrine disruptors on children with idiopathic precocious puberty. *Eur J Endocrinol* 2012: **166;** 803-809.

Dhondt MMM, Adriaens E, Remon JP. The evaluation of the local tolerance of vaginal formulations, with or without nonoxynol-9, using the slug mucosal irritation test. *Sex Transm Dis* 2004: **31**; 229-235.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev* 2009: **30;** 293-342.

Dohle GR. Male infertility in cancer patients: Review of the literature. *Int J Urol* 2010: **17;** 327-331.

Donnelly ET, McClure N, Lewis SE. Glutathione and hypotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species. *Mutagenesis* 2000: **15**; 61-68.

Donovan B. Barriers to conception and disease. *Ann Acad Med Singapore* 1995: **24**; 608-614.

Drevet JR. The antioxidant glutathione peroxidase family and spermatozoa: a complex story. *Mol Cell Endocrinol* 2006: **250**; 70-79.

Drife JO. The effects of drugs on sperm. *Drugs* 1987: **33;** 610-622.

Drobnis EZ, Yudin AI, Cherr GN, Katz DF. Hamster sperm penetration of the zona pellucida: kinematic analysis and mechanical implications. *Dev Biol* 1988: **130**; 311-323.

Dun MD, Aitken RJ, Nixon B. The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa. *Hum Reprod Update* 2012: **18;** 420-435.

Dunbar B, Patel M, Fahey J, Wira C. Endocrine control of mucosal immunity in the female reproductive tract: impact of environmental disruptors. *Mol Cell Endocrinol* 2012: **354;** 85-93.

ECETOC. Technical report 106: Guidance on identifying endocrine disrupting effects. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels. 2009: available at http://bit.ly/ecetoc-tr106 accessed 22 October 2012.

Edwards R, Steptoe P. A Matter of Life. 1980. Hutchinson and Co, London, UK.

Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization *in vitro* of human oocytes matured *in vitro*. *Nature* 1969: **221**; 632-635.

Edwards TM, Moore BC, Guillette LJ Jr. Reproductive dysgenesis in wildlife: a comparative view. *Int J Androl* 2006: **29;** 109-121.

Eliasson R. Semen analysis and laboratory workup. In Cockett ATK and Urry RL (eds) Male Infertility. Workup, Treatment and Research. 1977. Grune & Stratton, New York, USA, pp. 169-188.

Enciso M, Sarasa J, Agarwal A, Fernández JL, Gosálvez J. A two-tailed Comet assay for assessing DNA damage in spermatozoa. *Reprod Biomed Online* 2009: **18**; 609-616.

ESHRE Andrology Special Interest Group. Consensus workshop on advanced diagnostic andrology techniques. *Hum Reprod* 1996: **11**; 1463-1479.

ESHRE Andrology Special Interest Group. Guidelines on the application of CASA technology in the analysis of spermatozoa. *Hum Reprod* 1998: **13**; 142-145.

Eskenazi B, Wyrobek AJ, Fenster L, Katz DF, Sadler M, Lee J, Hudes M, Rempel DM. A study of the effect of perchloroethylene exposure on semen quality in dry cleaning workers. *Am J Ind Med* 1991: **20;** 575-591.

Esterhuizen AD, Franken DR, Becker PJ, Lourens JG, Müller II, van Rooyen LH. Defective sperm decondensation: a cause for fertilization failure. *Andrologia* 2002: **34;** 1-7.

European Science Foundation. Male Reproductive Health. Its impacts in relation to general wellbeing and low European fertility rates. Science Policy Briefing 40, 2010. http://www.esf.org/publications/science-policy-briefings.html accessed 22 October 2012.

Eustache F, Auger J. Inter-individual variability in the morphological assessment of human sperm: effect of the level of experience and the use of standard methods. *Hum Reprod* 2003: **18;** 1018-1022.

Eustache F, Auger J, Cabrol D, Jouannet P. Are volunteers delivering semen samples in fertility studies a biased population? *Hum Reprod* 2004: **19;** 2831-2837.

Evenson DP. Sperm chromatin strcuture assay (SCSA®). In Carrell DT and Aston KI (eds) Spermatogenesis: Methods and Protocols. *Methods in Molecular Biology* 2013: **927;** 147-164.

Evenson DP, Wixon R. Data analysis of two in vivo fertility studies using Sperm Chromatin Structure Assay-derived DNA fragmentation index vs. pregnancy outcome. *Fertil Steril* 2008: **90**; 1229-1231.

Fariello RM, Pariz JR, Spaine DM, Gozzo FC, Pilau EJ, Fraietta R, Bertolla RP, Andreoni C, Cedenho AP. Effect of smoking on the functional aspects of sperm and seminal plasma protein profiles in patients with varicocele. *Hum Reprod* 2012: **27;** 3140-3149.

Feneux D, Serres C, Jouannet P. Sliding spermatozoa: a dyskinesia responsible for human infertility? *Fertil Steril* 1985: **44**; 508-511.

Fernández JL, Cajigal D, Gosálvez J. Simultaneous labeling of single- and double-strand DNA breaks by DNA breakage detection-FISH (DBD-FISH). *Methods Mol Biol* 2011: **682**; 133-147.

Florman HM, Jungnickel MK, Sutton KA. Shedding light on sperm pHertility. *Cell* 2010: **140;** 310-312.

Forman R, Gilmour-White S, Forman N. Drug-Induced Infertility and Sexual Dysfunction. 1996. Cambridge University Press, Cambridge, UK.

Fraga CG, Motchnik PA, Shigenaga MK,Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci USA* 1991: **88**; 11003-11006.

Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* 1996: **351**; 199-203.

Franco JG Jr, Mauri AL, Petersen CG, Massaro FC, Silva LF, Felipe V, Cavagna M, Pontes A, Baruffi RL, Oliveira JB, Vagnini LD. Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa. *Int J Androl* 2012: **35;** 46-51.

Franken DR, Oehninger S, Burkman LJ, Coddington CC, Kruger TF, Rosenwaks Z, Acosta AA, Hodgen GD. The hemizona assays (HZA): a predictor of human sperm fertilizing potential in in vitro fertilization (IVF) treatment. *J In Vitro Fertil Embryo Transf* 1989: **6**; 44-49.

Franken DR, Smith M, Menkveld R, Kruger TF, Sekadde-Kigondu C, Mbizvo M, Akande EO. The development of a continuous quality control programme for strict sperm morphology among sub-Saharan African laboratories. *Hum Reprod* 2000: **15;** 667-671.

Fraser LR. Mouse sperm capacitation assessed by kinetics and morphology of fertilization in vitro. *J Reprod Fertil* 1983: **69;** 419-428.

Fraser LR. Sperm capacitation and the acrosome reaction. *Hum Reprod* 1998: **13 Suppl 1;** 9-19.

Frishman GN, Luciano AA, Maier DB. Evaluation of Astroglide, a new vaginal lubricant: effects of length of exposure and concentration on sperm motility. *Fertil Steril* 1992: **58**; 630-632.

Fronczak CM, Kim ED, Barqawi AB. The insults of recreational drug abuse on male fertility. *J Androl* Epub ahead of print 28 Jul 2011; doi: 10.2164/jandrol.110.011874.

Fuentes A, Muñoz A, Barnhart K, Argüello B, Díaz M, Pommer R. Recent cigarette smoking and assisted reproductive technologies outcome. *Fertil Steril* 2010: **93;** 89-95.

Gadea J, Molla M, Selles E, Marco MA, Garcia-Vazquez FA, Gardon JC. Reduced glutathione content in human sperm is decreased after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Cryobiology* 2011: **62**; 40-46.

Gadêlha H, Gaffney EA, Smith DJ, Kirkman-Brown JC. Nonlinear instability in flagellar dynamics: a novel modulation mechanism in sperm migration? *J R Soc Interface* 2010: **7**; 1689-1697.

Gadella BM, Tsai PS, Boerke A, Brewis IA. Sperm head membrane reorganisation during capacitation. *Int J Dev Biol* 2008: **52;** 473-480.

Gadella BM, Van Gestel RA. Bicarbonate and its role in mammalian sperm function. *Anim Reprod Sci* 2004: **82-83;** 307-319.

Gardner DK, Reed L, Linck D, Sheehan C, Lane M. Quality control in human in vitro fertilization. *Semin Reprod Med* 2005: **23;** 319-224.

Gelbke HP, Kayser M, Poole A. OECD test strategies and methods for endocrine disruptors. *Toxicology* 2004: **205**; 17-25.

Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* 2011: **26**; 1628-1640.

Gilmore JA, McGann LE, Liu J, Gao DY, Peter AT, Kleinhans FW, Critser JK. Effect of cryoprotectant solutes on water permeability of human spermatozoa. *Biol Reprod* 1995: **53**; 985-995.

Giwercman A. Estrogens and phytoestrogens in male infertility. *Curr Opin Urol* 2011: **21;** 519-526.

Glina S, Neves PA, Saade R, Netto NR Jr, Soares JB, Galuppo AG. Finasteride-associated male infertility. *Rev Hosp Clin Fac Med Sao Paulo* 2004: **59**;203-205.

Goldenburg RL, White R. The effect of vaginal lubricants on sperm motility in vitro. *Fertil Steril* 1975: **26**; 872-873.

Goldstein M, Eid JE. Elevation of intra-testicular temperature and scrotal skin surface temperature in men with varicocele. *J Urol* 1989: **142**; 743-745.

Gomez E, Aitken J. Impact of in vitro fertilization culture media on peroxidative damage to human spermatozoa. *Fertil Steril* 1996: **65**; 880-882.

Gomez E, Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. *Int J Androl* 1998: **21;** 81-94.

Gopalkrishnan K, Hinduja IN, Anand Kumar TC. Determining the osmolality of seminal fluid aids in the rapid diagnosis of the fertilizing potential of spermatozoa. *Assist Reprod Genet* 1989: **6**; 119-121.

Gore AC. Endocrine-disrupting chemicals: From basic research to clinical practice. 2007. Humana Press, Totowa, NJ, USA.

Gore AC, Walker DM, Zama AM, Armenti AE, Uzumcu M. Early life exposure to endocrine-disrupting chemicals causes lifelong molecular reprogramming of the hypothalamus and premature reproductive aging. *Mol Endocrinol* 2011: **25;** 2157-2168.

Gosden RG. Oogenesis as a foundation for embryogenesis. *Mol Cell Endocrinol* 2002: **186**; 149-153.

Gottardo F, Kliesch S. Ejakulatdiagnostik. Spermiogramm nach WHO-Kriterien 2010 *Der Urologe* 2011: **50;** 101-108.

Griveau JF, Lobel B, Laurent MC, Michardiere L, Le Lannou D. Interest of pentoxifylline in ICSI with frozen-thawed testicular spermatozoa from patients in non-obstructive azoospermia. *Reprod Biomed Online* 2006: **12**; 14-18.

Gruber CJ, Gruber DM, Gruber IM, Wieser F, Huber JC. Anatomy of the estrogen response element. *Trends Endocrinol Metab* 2004: **15;** 73-78.

Guerrero A, Carneiro J, Pimentel A, Wood CD, Corkidi G, Darszon A. Strategies for locating the female gamete: the importance of measuring sperm trajectories in three spatial dimensions. *Mol Hum Reprod* 2011: **17;** 511-523.

Haimov-Kochman R, Ben-Chetrit E. The effect of colchicine treatment on sperm production and function: a review. *Hum Reprod* 1998: **13;** 360-362.

Hakimi H, Geary I, Pacey A, Eley A. Spermicidal activity of bacterial lipopolysaccharide is only partly due to lipid A. *J Androl* 2006: **27**; 774-779.

Hamlin HJ Guillette LJ. Birth defects in wildlife: the role of environmental contaminants as inducers of reproductive and developmental dysfunction. *Sys Biol Reprod Med* 2010: **56;** 113-121.

Hargreave TB (ed). Male infertility, 2nd edition. 1994a. Springer-Verlag, Berlin, Germany.

Hargreave TB. Varicocele. In Hargreave TB (ed), Male infertility, 2nd edition. 1994b. Springer-Verlag, Berlin, Germany, pp. 249-267.

Harris RM, Waring RH. Diethylstilboestrol – a long-term legacy. *Maturitas* 2012: **72;** 108-112.

Harvey PW, Everett DJ. Regulation of endocrine-disrupting chemicals: Critical overview and deficiencies in toxicology and risk assessment for human health. *Best Pract Res Clin Endocrinol Metab* 2006: **20**; 145-165.

He Z, Kokkinaki M, Pant D, Gallicano GI, Dym M. Small RNA molecules in the regulation of spermatogenesis. *Reproduction* 2009: **137;** 901-911.

Henkel RR. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian J Androl* 2011: **13**; 43-52.

Henkel RR, Schill WB. Sperm preparation for ART. *Reprod Biol Endocrinol* 2003: **1**; 108.

Hermo L, Pelletier RM, Cyr DG, Smith CE. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. *Microsc Res Tech* 2010a: **73;** 241-278.

Hermo L, Pelletier RM, Cyr DG, Smith CE. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 2: changes in spermatid organelles associated with development of spermatozoa. *Microsc Res Tech* 2010b: **73;** 279-319.

Hermo L, Pelletier RM, Cyr DG, Smith CE. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 3: developmental changes in spermatid flagellum and cytoplasmic droplet and interaction of sperm with the zona pellucida and egg plasma membrane. *Microsc Res Tech* 2010: **73;** 320-363.

Ho HC, Suarez SS. Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biol Reprod* 2003: **68**; 1590-1596.

Holland MK, Alvarez JG, Storey BT. Production of superoxide and activity of superoxide dismutase in rabbit epididymal spermatozoa. *Biol Reprod* 1982: **27**; 1109-1118.

Hong CY, Chaput de Saintonge DM, Turner P. A simple method to measure drug effects on human sperm motility. *Br J Clin Pharmacol* 1981a: **11;** 385-387.

Hong CY, Chaput de Saintonge DM, Turner P. The inhibitory action of procaine, (+)-propranolol and (+/-)-propranolol on human sperm motility: antagonism by caffeine. *Br J Clin Pharmacol* 1981b: **12;** 751-753.

Hong CY, Chaput de Saintonge DM, Turner P. Effects of chlorpromazine and other drugs acting on the central nervous system on human sperm motility. *Eur J Clin Pharmacol* 1982: **22;** 413-416.

Holt WV, Fazeli A. The oviduct as a complex mediator of mammalian sperm function and selection. *Mol Reprod Dev* 2010: **77**; 934-943.

Huang LP, Lee CC, Hsu PC, Shih TS. The association between semen quality in workers and the concentration of di(2-ethylhexyl) phthalate in polyvinyl chloride pellet plant air. *Fertil Steril* 2011: **96;** 90-94.

Hunter RH, Coy P, Gadea J, Rath D. Considerations of viscosity in the preliminaries to mammalian fertilisation. *J Assist Reprod Genet* 2011: **28;** 191-197.

Ikawa M, Inoue N, Benham AM, Okabe M. Fertilization: a sperm's journey to and interaction with the oocyte. *J Clin Invest* 2010: **120**; 984-994.

Inaba K. Sperm flagella: comparative and phylogenetic perspectives of protein components. *Mol Hum Reprod* 2011: **17;** 524-538.

Inoue K, Kondo S, Yoshie Y, Kato K, Yoshimura Y, Horie M, Nakazawa H. Migration of 4-nonylphenol chloride food packaging films into food simulants and foods. *Food Addit Contam* 2001: **18**; 157-164.

International Organization for Standardization. ISO 10993-10:2010 Biological evaluation of medical devices Part 10: Tests for irritation and delayed-type hypersensitivity. 2010. International Organization for Standardization, Geneva, Switzerland.

Irwig MS, Kolukula S. Persistent sexual side effects of finasteride for male pattern hair loss. *J Sex Med* 2011: **8**;1747-1753.

Ishijima S. Dynamics of flagellar force generated by a hyperactivated spermatozoon. *Reproduction* 2011: **142;** 409-415.

Isobe T. New method to estimate the possibility of natural pregnancy using computer-assisted sperm analysis. *Syst Biol Reprod Med* 2012 Epub ahead of print doi:10.3109/19396368.2012.700759

Isojärvi JI, Löfgren E, Juntunen KS, Pakarinen AJ, Päivänsalo M, Rautakorpi I, Tuomivaara L. Effect of epilepsy and antiepileptic drugs on male reproductive health. *Neurology* 2004: **62;** 247-253.

Ivic A, Onyeaka H, Girling A, Brewis IA, Ola B, Hammadieh N, Papaioannou S, Barratt CL. Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Hum Reprod* 2002: **17**; 143-149.

Jääskeläinen EL, Teplova V, Andersson MA, Andersson LC, Tammela P, Andersson MC, Pirhonen TI, Saris NE, Vuorela P, Salkinoja-Salonen MS. In vitro assay for human toxicity of cereulide, the emetic mitochondrial toxin produced by food poisoning Bacillus cereus. *Toxicol In Vitro* 2003: **17;** 737-744.

Jensen TK, Jørgensen N, Punab M, Haugen TB, Suominen J, Zilaitiene B, Horte A, Andersen AG, Carlsen E, Magnus Ø *et al.* Association of in utero exposure to maternal smoking with reduced semen quality and testis size in adulthood: a cross-sectional study of 1,770 young men from the general population in five European countries. *Am J Epidemiol* 2004: **159;** 49-58.

Jequier AM. Male Infertility: A Clinical Guide. 2011. Cambridge University Press, Cambridge, UK.

Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. Development of an assay to assess the functional integrity of the human

sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 1984: **70**; 219-228.

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc Natl Acad Sci USA* 2011: **108**; 4892-4896.

Jobling MS, Hutchison GR, van den Driesche S, Sharpe RM. Effects of di(n-butyl) phthalate exposure on foetal rat germ-cell number and differentiation: identification of age-specific windows of vulnerability. *Int J Androl* 2011: **34;** e386-396.

Joffe M. What has happened to human fertility? *Hum Reprod* 2010: **25;** 295-307.

Johnson L, Petty CS, Neaves WB. A comparative study of daily sperm production and testicular composition in humans and rats. *Biol Reprod* 1980: **22**; 1233-1243.

Johnston DE, Chiao Y, Gaveler JS, Van Weil DH. Inhibition of testosterone synthesis by ethanol and acetaldehyde. *Biochem Pharmacol* 1981: **30**; 1827-1831.

Jones RC. To store or mature spermatozoa? The primary role of the epididymis. *Int J Androl* 1999: **22;** 57-67.

Jouannet P, Ducot B, Feneux D, Spira F. Male factors and the likelihood of pregnancy in infertile couples. I. Study of sperm characteristics. *Int J Androl* 1988: **11;** 379–394.

Jurewicz J, Hanke W, Radwan M, Bonde JP. Environmental factors and semen quality. *Int J Occup Med Environ Health* 2009: **22;** 305-329.

Kabuto H, Amakawa M, Shishibori T. Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. *Life Sci* 2004: **74;** 2931-2940.

Kacem O, Sifer C, Barraud-Lange V, Ducot B, De Ziegler D, Poirot C, Wolf J. Sperm nuclear vacuoles, as assessed by motile sperm organellar morphological examination, are mostly of acrosomal origin. *Reprod Biomed Online* 2010: **20;** 132-137.

Kalfa N, Philibert P, Sultan C. Is hypospadias a genetic, endocrine or environmental disease, or still an unexplained malformation? *Int J Androl* 2009: **32;** 187-197.

Kang W, Zhou C, Koga Y, Baba T. Hyaluronan-degrading activity of mouse sperm hyaluronidase is not required for fertilization? *J Reprod Dev* 2010: **56**; 140-144.

Kanwar KC, Yanagimachi R, Lopata A. Effects of human seminal plasma on fertilizing capacity of human spermatozoa. *Fertil Steril* 1979: **31**; 321-327.

Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 2010: **16;** 690-703.

Katkov II, Katkova N, Critser JK, Mazur P. Mouse spermatozoa in high concentrations of glycerol: chemical toxicity vs osmotic shock at normal and reduced oxygen concentrations. *Cryobiology* 1998: **37**; 325-338.

Kefer JC, Agarwal A, Sabanegh E. Role of antioxidants in the treatment of male infertility. *Int J Urol* 2009: **16**; 449-457.

Keinan-Boker L, van Der Schouw YT, Grobbee DE, Peeters PH. Dietary phytoestrogens and bresat cancer risk. *Am J Clin Nutr* 2004: **79;** 282-288.

Kirchin VS, Southgate HJ, Beard RC. Colchicine: an unusual cause of reversible azoospermia. *BJU Int* 1999: **83;** 156.

Kirkman-Brown JC, Smith DJ. Sperm Motility: is viscosity fundamental to progress? *Mol Hum Reprod* 2011: **17**; 539-544.

Klinefelter GR, Laskey JW, Winnik WM, Suarez JD, Roberts NL, Strader LF, Riffle BW, Veeramachaneni DN. Novel molecular targets associated with testicular dysgenesis induced by gestational exposure to diethylhexyl phthalate in the rat: a role for estradiol. *Reproduction* 2012: **144;** 747-761

Kolodny RC, Master WH, Kolodney AB, Toro G. Depression of plasma testosterone levels after chronic intensive marijuana use. *New Engl J Med* 1974: **290**; 872-874.

Kortenkamp A. Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. *Environ Health Perspect* 2007: **115 Suppl 1;** 98-105.

Kortenkamp A, Faust M, Scholze M, Backhaus T. Low-level exposure to multiple chemicals: reason for human health concerns? *Environ Health Perspect* 2007: **115 Suppl 1;** 106-114.

Kortenkamp A, Martin O, Faust M, Evans R, McKinlay R, Orton F, Rosivatz E. State of the art assessment of endocrine disrupters. European Commission Report 2012: available at http://ec.europa.eu/environment/endocrine/documents/4_SOTA%20EDC%20Final%20Report%20V3%206%20Feb%2012.pdf, accessed 22 October 2012.

Kosova G, Scott NM, Niederberger C, Prins GS, Ober C. Genome-wide association study identifies candidate genes for male fertility traits in human. *Am J Hum Genet* 2012: **90;** 950-961.

Krisher RL. The effect of oocyte quality on development. *J Anim Sci* 2004: **82 E-Suppl**; E14-23.

Kristensen DM, Hass U, Lesné L, Lottrup G, Jacobsen PR, Desdoits-Lethimonier C, Boberg J, Petersen JH, Toppari J, Jensen TK, *et al.* Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. *Hum Reprod* 2011: **26;** 235-244.

Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van Der Merwe JP, Van Zyl JA, Smith K. Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril* 1986: **46;** 1118–1123.

Kuhl AJ, Ross SM, Gaido KW. CCAAT/enhancer binding protein beta, but not steroidogenic factor-1, modulates the phthalate-induced dysregulation of rat fetal testicular steroidogenesis. *Endocrinology* 2007: **148;** 5851-5864.

Kumar D, Kalthur G, Mascarenhas C, Kumar P, Adiga SK. Ejaculate fractions of asthenozoospermic and teratozoospermic patients have

differences in the sperm DNA integrity. *Andrologia* 2011: **43**; 416-421.

Kumar N, Jain S, Gupta A, Tiwary AK. Spermicidal activity of sulfonylureas and meglitinide analogues: role of intrasperm Ca^{2+} elevation. *J Pharm Pharmacol* 2008: **60**; 323-330.

Kutteh WH, Choe CH, Ritter JO, Byrd W. Vaginal lubricants for the infertile couple: effect on sperm activity. *Int J Fertil Menopause Stud* 1996: **41**; 400-404.

Kvist U, Björndahl L. Zinc preserves an inherent capacity for human sperm chromatin decondensation. *Acta Physiol Scand* 1985: **124**; 195-200.

Kvist U, Björndahl L, Roomans GM, Lindholmer C. Nuclear zinc in human epididymal and ejaculated spermatozoa. *Acta Physiol Scand* 1985: **125**; 297-303.

Laier P, Metzdorff SB, Borch J, Hagen ML, Hass U, Christiansen S, Axelstad M, Kledal T, Dalgaard M, McKinnell C, et al. Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicol Appl Pharmacol* 2006: **213;** 160-171.

Lambrot R, Muczynski V, Lécureuil C, Angenard G, Coffigny H, Pairault C, Moison D, Frydman R, Habert R, Rouiller-Fabre V. Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. *Environ Health Perspect* 2009: **117;** 32-37.

Lass A, Akagbosu F, Brinsden P. Sperm banking and assisted reproduction treatment for couples following cancer treatment of the male partner. *Hum Reprod Update* 2001: **7**; 370-377

Latini G, Del Vecchio A, Massaro M, Verrotti A, De Felice C. Phthalate exposure and male infertility. *Toxicology* 2006: **226**; 90-98.

Lazzari G, Tessaro I, Crotti G, Galli C, Hoffmann S, Bremer S, Pellizzer C. Development of an in vitro test battery for assessing chemical effects on bovine germ cells under the ReProTect umbrella.*Toxicol Appl Pharmacol* 2008: **233;** 360-370.

Leahy T, Gadella BM. Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction* 2011: **142;** 759-778.

Lee J, Choe E. Effects of phosphatidylcholine and phosphatidylethanolamine on the photooxidation of canola oil. *J Food Sci* 2009: **74**; C481-486.

Lee MA, Trucco GS, Bechtol KB, Wummer N, Kopf GS, Blasco L, Storey BT. Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay. *Fertil Steril* 1987: **48**; 649-658.

Levi AJ, Fisher AM, Hughes L, Hendry WF. Male infertility due to sulphasalazine. *Lancet* 1979: **ii**; 276-278.

Levin RM, Amsterdam JD, Winokur A, Wein AJ. Effects of psychotropic drugs on human sperm motility. *Fertil Steril* 1981: **36;** 503-506.

Levin RJ. Can the controversy about the putative role of the human female orgasm in sperm transport be settled with our current physiological knowledge of coitus? *J Sex Med* 2011: **8**; 1566–1578.

Lewis SE, Simon L. Clinical implications of sperm DNA damage. *Hum Fertil* 2010: **13**; 201-207.

Lindemann CB, Lesich KA. Flagellar and ciliary beating: the proven and the possible. *J Cell Sci* 2010: 123; 519-528.

Lindholmer C. Survival of human spermatozoa in different fractions of split ejaculate. *Fertil Steril* 1973: **24**; 521-526.

Lindholmer C, Eliasson R. The effects of albumin, magnesium, and zinc on human sperm survival in different fractions of split ejaculates. *Fertil Steril* 1974: **25**; 424-431.

Linschooten JO, Laubenthal J, Cemeli E, Baumgartner A, Anderson D, Sipinen VE, Brunborg G, Haenen GR, Fthenou E, Briedé JJ *et al.* Incomplete protection of genetic integrity of mature spermatozoa against oxidative stress. *Reprod Toxicol* 2011: **32;** 106-111.

Lishko PV, Kirichok Y, Ren D, Navarro B, Chung JJ, Clapham DE. The control of male fertility by spermatozoan ion channels. *Ann Rev Physiol* 2012: **74;** 453-475.

Liu DY, Baker HWG. Morphology of spermatozoa bound to the zona pellucida of human oocytes that failed to fertilize in vitro. *J Reprod Fertil* 1992: **94;** 71–84.

Liu DY, Baker HWG. Tests for human sperm zona pellucida binding and penetration. In: Tesarik J (ed), Male Factor in Human Infertility. Frontiers in Endocrinology, Vol. 8. 1994. Ares Serono Symposia, Rome, pp. 169-185.

Liu DY, Garrett C, Baker HW. Acrosome-reacted human sperm in insemination medium do not bind to the zona pellucida of human oocytes. *Int J Androl* 2006: **29;** 475-481.

Liu DY, Lopata A, Johnston WIH, Baker HWG. A human sperm-zona pellucida binding test using oocytes that failed to fertilize in vitro. *Fertil Steril* 1988: **50**; 782-788.

Liu K, Lehmann KP, Sar M, Young SS, Gaido KW. Gene expression profiling following in utero exposure to phthalate esters reveals new gene targets in the etiology of testicular dysgenesis. *Biol Reprod* 2005: **73;** 180-192.

Lynch E, Braithwaite R. A review of the clinical and toxicological aspects of 'traditional' (herbal) medicines adulterated with heavy metals. *Expert Opin Drug Safety* 2005: **4;** 769-78.

MacLeod J, Gold RZ. The male factor in fertility and infertility. III. An analysis of motile activity in the spermatozoa of 1000 fertile men and 1000 men in infertile marriage. *Fertil Steril* 1951: **2**; 187-204.

MacLeod J, Hotchkiss RS, Sitterson BW. Recovery of male infertility after sterilisation by nuclear radiation. *JAMA* 1964: **187**; 637-642.

Mahmoud AM, Depoorter B, Piens N, Comhaire FH. The performance of 10 different methods for the estimation of spermconcentration. *Fertil Steril* 1997: **68;** 340-345.

Makker K, Varghese A, Desai NR, Mouradi R, Agarwal A. Cell phones: modern man's nemesis? *Reprod Biomed Online* 2009: **18**; 148-157.

Makler A, Deutch M, Vilensky A, Palti Y. Factors affecting sperm motility VIII. Velocity and survival of human spermatozoa as related to

temperatures above zero. *Int J Androl* 1981: **4**; 559-569.

Makler A, Tatcher M, Vilensky A, Brandes JM. Factors affecting sperm motility. III. Influence of visible light and other electromagnetic radiations on human sperm velocity and survival. *Fertil Steril* 1980: **33**; 439-444.

Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. *PLoS One* 2012a: **7;** e46249.

Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Pesticide and insect repellent mixture (Permethrin and DEET) Induces epigenetic transgenerational inheritance of disease and sperm epimutations. *Reprod Toxicol* 2012b: doi: 10.1016/j.reprotox.2012.08.010. [Epub ahead of print]

Mann T. The Biochemistry of Semen and of the Male Reproductive Tract. 1964. Methuen and Co, London, UK.

MarketResearch.com. Cosmetics & Personal Care Market Research Reports. www.marketresearch.com/Consumer-Goods-c1596/Consumer-Goods-Retailing-c80/Cosmetics-Personal-Care-c113/, accessed 22 October 2012.

Mariappa D, Siva AB, Shivaji S, Seshagiri PB. Tyrphostin-A47 inhibitable tyrosine phosphorylation of flagellar proteins is associated with distinct alteration of motility pattern in hamster spermatozoa. *Mol Reprod Dev* 2006: **73;** 215-225.

Marques-Mari AI, Lacham-Kaplan O, Medrano JV, Pellicer A, Simón C. Differentiation of germ cells and gametes from stem cells. *Hum Reprod Update* 2009: **15;** 379-390.

Martin CW, Anderson RA, Cheng L. Potential impact of hormonal male contraception: cross-cultural implications for the development of novel preparations. *Hum Reprod* 2000: **15**; 637-645.

Martin RH. Cytogenetic determinants of male fertility. *Hum Reprod Update* 2008: **14;** 379-390.

Martin-DeLeon PA, Boice ML. Sperm aging in the male and cytogenetic anomalies. An animal model. *Hum Genet* 1982: **62**; 70-77.

Martin-Deleon PA, Shaver EL, Gammal EB. Chromosome abnormalities in rabbit blastocysts resulting from spermatozoa aged in the male tract. *Fertil Steril* 1973: **24**; 212-219.

Marx-Stoelting P, Pfeil R, Solecki R, Ulbrich B, Grote K, Ritz V, Banasiak U, Heinrich-Hirsch B, Moeller T, Chahoud I, Hirsch-Ernst KI. Assessment strategies and decision criteria for pesticides with endocrine disrupting properties relevant to humans. *Reprod Toxicol* 2011: **31;** 574-584.

Marzec-Wróblewska U, Kamiński P, Lakota P. Influence of chemical elements on mammalian spermatozoa. *Folia Biol (Praha)* 2012: **58;** 7-15.

Matson PL, Yovich JM, Edirisinghe WR, Junk SM, Yovich JL. An argument for the past and continued use of pentoxifylline in assisted reproductive technology. *Hum Reprod* 1995: **10 Suppl 1**: 67-71.

McCarrey JR. The epigenome as a target for heritable environmental disruptions of cellular function. *Mol Cell Endocrinol* 2012: 354; 9-15.

McClusky LM, de Jager C, Bornman MS. Stage-related increase in the proportion of apoptotic germ cells and altered frequencies of stages in the spermatogenic cycle following gestational, lactational and direct exposure of male rats to p-nonylphenol. *Toxicol Sci* 2007: **95**; 249-256.

McIver SC, Roman SD, Nixon B, McLaughlin EA. miRNA and mammalian male germ cells. *Hum Reprod Update* 2012: **18;** 44-59.

McLachlan RI, O'Bryan MK. State of the art for genetic testing of infertile men. *J Clin Endocrinol Metab* 2010: **95**; 1013-1024.

Menkveld R, Stander FSH, Kotze TJvW, Kruger TF, Van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod* 1990: **5;** 586–592.

Merzenich H, Zeeb H, Blettner M. Decreasing sperm quality: a global problem? *BMC Public Health* 2010: **10;** 24.

Mieusset R, Quintna Casares P, Sanchez Partida LP, Sowerbutts SF, Zipp JL, Setchell BP. Effects of heating the epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. *J Reprod Fertil* 1992: **94**; 337-343.

Miller B, Klein TA, Opsahl MS. The effect of surgical lubricant on in vivo sperm penetration of cervical mucus. *Fertil Steril* 1994: **6**; 1171-1173.

Mitchell RT, Saunders PT, Sharpe RM, Kelnar CJ, Wallace WH. Male fertility and strategies for fertility preservation following childhood cancer treatment. *Endocr Dev* 2009: **15;** 101-134.

Mnif W, Hassine AID, Bouaziz A, Bartegi A, Thomas O, Roig B. Effect of endocrine disruptor pesticides: a review. *Int J Environ Res Public Health* 2011: **8;** 2265-2303.

Moline J, Golden A, Bar-Chama N, Smith E, Rauch ME, Chapin RE, Perreault SD, Schrader SM, Suk WA, Landrigan PJ. Exposure to hazardous substances and male reproductive health: a research framework. *Environ Health Perspect* 2000: **108**; 803-813.

Montjean D, Belloc S, Benkhalifa M, Dalleac A, Ménézo Y. Sperm vacuoles are linked to capacitation and acrosomal status. *Hum Reprod* 2012: **27;** 2927-2932.

Moosani N, Pattinson HA, Carter MD, Cox DM, Rademaker AW, Martin RH. Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence in situ hybridization. *Fertil Steril* 1995: **64**; 811-817.

Morbeck DE, Khan Z, Barnidge DR, Walker DL. Washing mineral oil reduces contaminants and embryotoxicity. *Fertil Steril* 2010: **94**; 2747-2752.

Morgan DJ, Muller CH, Murataeva NA, Davis BJ, Mackie K. Delta-9-tetrahydrocannabinol (Δ(9)-THC) attenuates mouse sperm motility and male fecundity. *Br J Pharmacol* 2012: **165**; 2575-2583.

Mortimer D. Sperm transport in the human female reproductive tract. In Finn CA (ed) Oxford Reviews of Reproductive Biology, Vol. 5. 1983. Oxford University Press, Oxford, UK, pp. 30-61.

Mortimer D. Elaboration of a new culture medium for physiological studies on human sperm motility and capacitation. *Hum Reprod* 1986: **1**; 247-250.

Mortimer D. Practical Laboratory Andrology. 1994. Oxford University Press, New York, USA.

Mortimer D. *In vitro* induction of the human sperm acrosome reaction. In Fénichel P and Parinaud J (eds) Human Sperm Acrosome Reaction. *Colloque INSERM/John Libbey Eurotext Ltd, Montrouge* 1995a: **236**; 295-313.

Mortimer D. Sperm transport in the female genital tract. In Grudzinskas JG and Yovich JL (eds) Cambridge Reviews in Human Reproduction, Volume 2: Gametes – The Spermatozoon. 1995b. Cambridge University Press, Cambridge, UK, pp. 157-174.

Mortimer D. Sperm preparation methods. *J Androl* 2000: **21**; 357-66.

Mortimer D. Sperm form and function: Beauty is in the eye of the beholder. In van der Horst G, Franken D, Bornman R, de Jager T, Dyer S (eds) Proceedings of 9th International Symposium on Spermatology. 2002. Monduzzi Editore, Bologna, Italy, pp. 257-262.

Mortimer D. Essential features of media development for spermatozoa, oocytes and embryos. In Quinn P (ed) Culture Media, Solutions & Systems in Human ART. 2013. Cambridge University Press, Cambridge, UK, in press.

Mortimer D, Menkveld R. Sperm morphology assessment – historical perspectives and current opinions. *J Androl* 2001: **22;** 192-205.

Mortimer D, Aitken RJ, Mortimer ST, Pacey AA. Clinical CASA – The quest for consensus. *Reprod Fertil Dev* 1995: **7**; 951-959.

Mortimer D, Curtis EF, Camenzind AR. Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. *Hum Reprod* 1990: **5**; 99-103.

Mortimer D, Curtis EF, Camenzind AR, Tanaka S. The spontaneous acrosome reaction of human spermatozoa incubated in vitro. *Hum Reprod* 1989: **4**; 57-62.

Mortimer D, Mortimer ST. Value and reliability of CASA systems. In Ombelet W, Bosmans E, Vandeput H, Vereecken A, Renier M, Hoomans E (eds) Modern ART in the 2000s – Andrology in the Nineties. 1998. Parthenon, New York/London, pp. 73-89.

Mortimer D, Mortimer ST. Laboratory investigation of the infertile male. In Brinsden PR (ed) Textbook of In Vitro Fertilization and Assisted Reproduction. The Bourn Hall Guide to Clinical and Laboratory Practice. 2005. Taylor & Francis, Abingdon, UK, pp. 61-91.

Mortimer D, Mortimer ST. Computer-aided sperm analysis (CASA) of sperm motility and hyperactivation. In Carrell DT and Aston KI (eds) Spermatogenesis: Methods and Protocols. *Methods in Molecular Biology* 2013: **927;** 77-87.

Mortimer D, Mortimer ST, Shu MA, Swart R. A simplified approach to sperm-cervical mucus interaction using a hyaluronate migration test. *Hum Reprod* 1990: **5**; 835-841.

Mortimer D, Templeton AA. Sperm transport in the human female reproductive tract in relation to semen analysis characteristics and time of ovulation. *J Reprod Fertil* 1982: **64**; 401-408.

Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. *Hum Reprod Update* 1997: **3**; 403-39.

Mortimer ST. CASA – practical aspects. *J Androl* 2000: **21**; 515-524.

Moseley FL, Jha KN, Björndahl L, Brewis IA, Publicover SJ, Barratt CL, Lefièvre L. Protein tyrosine phosphorylation, hyperactivation and progesterone-induced acrosome reaction are enhanced in IVF media: an effect that is not associated with an increase in protein kinase A activation. *Mol Hum Reprod* 2005: **11**; 523-529.

Mouritsen A, Aksglaede L, Sørensen K, Mogensen SS, Leffers H, Main K M, Frederiksen H, Andersson AM, Skakkebaek NE, Juul, A. Hypothesis: exposure to endocrine-disrupting chemicals may interfere with timing of puberty. *Int J Androl* 2010: **33;** 346-359.

Muller CH, Ravnik SE. Lipid Transfer Protein: A natural stimulator of the sperm capacitation process. In Fénichel P and Parinaud J (eds) Human Sperm Acrosome Reaction. *Colloque INSERM/John Libbey Eurotext Ltd, Montrouge* 1995: **236**; 67-84.

Muncke J. Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source? *Sci Total Environ* 2009: **407**; 4549-4559.

Murialdo G, Galimberti CA, Fonzi S, Manni R, Costelli P, Parodi C, Torre F, Solinas GP, Polleri A, Tartara A. Sex hormones, gonadotropins and prolactin in male epileptic subjects in remission: role of the epileptic syndrome and of antiepileptic drugs. *Neuropsychobiology* 1994: **30**; 29-36.

Mutoh J, Taketoh J, Okamura K, Kagawa T, Ishida T, Ishii Y, Yamada H. Fetal pituitary gonadotropin as an initial target of dioxin in its impairment of cholesterol transportation and steroidogenesis in rats. *Endocrinology* 2006: **147;** 927-936.

National Research Council (U.S.). Committee on Toxicity Testing and Assessment of Environmental Agents. Toxicity testing for assessment of environmental agents interim report. 2006. National Academies Press (U.S.), Washington DC, USA.

Ndebele K, Graham B, Tchounwou PB. Estrogenic activity of coumestrol, DDT, and TCDD in human cervical cancer cells. *Int J Environ Res Public Health* 2010: **7;** 2045-2056.

Newbold RR. Developmental exposure to endocrine-disrupting chemicals programs for reproductive tract alterations and obesity later in life. *Am J Clin Nutr* 2011: **94;** 1939S-1942S.

Nieschlag E, Behre HM, Nieschlag S (eds). *Andrology: Male Reproductive Health and Dysfunction*, 3rd edition. 2010a. Springer-Verlag, Berlin, Germany.

Nieschlag E, Behre HM, Wieacker P, Meschede D, Kamischke A, Kliesch S. Disorders at the testicular level. In Nieschlag E, Behre HM, Nieschlag S (eds). *Andrology: Male Reproductive Health and Dysfunction*, 3rd edition. 2010b. Springer-Verlag, Berlin, Germany, pp. 193-238.

Oatley JM, Brinster RL. The germline stem cell niche unit in mammalian testes. *Physiol Rev* 2012: **92;** 577-595.

Oehninger S, Franken DR, Sayed E, Barroso G, Kolm P. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta-analysis. *Hum Reprod Update* 2000: **6**; 160-168.

Oliveira JB, Massaro FC, Baruffi RL, Mauri AL, Petersen CG, Silva LF, Vagnini LD, Franco JG Jr. Correlation between semen analysis by motile sperm organelle morphology examination and sperm DNA damage. *Fertil Steril* 2010: **94;** 1937-1940.

Olson SD, Fauci LJ, Suarez SS. Mathematical modeling of calcium signaling during sperm hyperactivation. *Mol Hum Reprod* 2011a: **17;** 500-510.

Olson SD, Suarez SS, Fauci LJ. A model of CatSper channel mediated calcium dynamics in mammalian spermatozoa. *Bull Math Biol* 2010: **72**; 1925-1946.

Olson SD, Suarez SS, Fauci LJ. Coupling biochemistry and hydrodynamics captures hyperactivated sperm motility in a simple flagellar model. *J Theor Biol* 2011b: **283;** 203-216.

Olshan AF, Perreault SD, Bradley L, Buus RM, Strader LF, Jeffay SC, Lansdell L, Savitz DA, Herring A. The healthy men study: design and recruitment considerations for environmental epidemiologic studies in male reproductive health. *Fertil Steril* 2007: **87;** 554-564.

Orta G, Ferreira G, José O, Treviño CL, Beltrán C, Darszon A. Human spermatozoa possess a calcium-dependent chloride channel that may participate in the acrosomal reaction. *J Physiol* 2012: **590;** 2659-2675.

Otsuki J, Nagai Y, Chiba K. Peroxidation of mineral oil used in droplet culture is detrimental to fertilization and embryo development. *Fertil Steril* 2007: **88**; 741-743.

Overstreet JW, Fuh VL, Gould J, Howards SS, Lieber MM, Hellstrom W, Shapiro S, Carroll P, Corfman RS, Petrou S, Lewis R, Toth P, Shown T, Roy J, Jarow JP, Bonilla J, Jacobsen CA, Wang DZ, Kaufman KD.Chronic treatment with finasteride daily does not affect spermatogenesis or semen production in young men. *J Urol* 1999: **162**;1295-1300.

Ozgur K, Franken DR, Kaskar K, Gabriel LK, Kruger TF. The influence of a mineral oil overlay on the zona pellucida binding potential of human spermatozoa. *Andrologia* 1995: **27**; 155-159.

Pacey AA. Environmental and lifestyle factors associated with sperm DNA damage. *Hum Fertil* 2010: **13**; 189-193.

Parazzini F, Marchini M, Luchini L, Tozzi L, Mezzopane R, Fedele L. Tight underpants and trousers and risk of dyspermia. *Int J Androl* 1995: **18;** 137-140.

Patton PE, Battaglia DE (eds). *Office Andrology.* 2005. Humana Press, Totowa, NJ, USA.

Pedersen H. Ultrastructure of the ejaculated human sperm. *Z Zellforsch Mikrosk Anat* 1969: **94**; 542-554.

Pedersen H, Fawcett DW. Functional anatomy of the human spermatozoon. In Hafez ESE (ed) Human Semen and Fertility Regulation in Men. 1976. CV Mosby Co, St Louis, USA, pp. 65-75.

Peng BC, Tomashevsky P, Nagler HM. The co-factor effect: varicocele and infertility. *Fertil Steril* 1990: **54**; 143-148.

Perdrix A, Travers A, Chelli MH, Escalier D, Do Rego JL, Milazzo JP, Mousset-Siméon N, Macé B, Rives N. Assessment of acrosome and nuclear abnormalities in human spermatozoa with large vacuoles. *Hum Reprod* 2011: **26;** 47-58.

Perry MJ. Effects of environmental and occupational pesticide exposure on human sperm: a systematic review. *Hum Reprod Update* 2008: **14**; 233-242.

Perry MJ, Venners SA, Chen X, Liu X, Tang G, Xing H, Barr DB, Xu X. Organophosphorous pesticide exposures and sperm quality. *Reprod Toxicol* 2011: **31**; 75-79.

Petzoldt R, Wolf H, Reefschläger J, Karge M, Berger G. A rapid quantitative method based on motility of bull sperm cells for in vitro toxicity testing of biomaterials. *Biomaterials* 1985: **6;** 105-109.

Phillips KP, Foster, WG. Key Developments in endocrine disrupter research and human health. *J Toxicol Environ Health B Crit Rev* 2008: **11**; 322-344.

Phillips KP, Tanphaichitr N. Human exposure to endocrine disrupters and semen quality. *J Toxicol Environ Health B Crit Rev* 2008: **11**; 188-220.

Phillips BT, Gassei K, Orwig KE. Spermatogonial stem cell regulation and spermatogenesis. *Phil Trans R Soc Lond B Biol Sci* 2010: **365;** 1663-1678.

Pinto B, Reali D. Screening of estrogen-like activity of mineral water stored in PET bottles. *Int J Hyg Environ Health* 2009: **212**; 228-232.

Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. *Int J Androl* 2012: **35;** 109-124.

Plymate JS, Tenover JS, Bremner WJ. Circadian variation in testosterone, sex hormone binding globulin and calculated non-sex hormone binding globulin bound testosterone secretion in healthy young and elderly men. *J Androl* 1989: **10**; 366-371.

Poças MdF, Hogg T. Exposure assessment of chemicals from packaging materials in foods: a review. *Trends Food Sci Tech* 2007: **18**; 219-230.

Publicover SJ, Giojalas LC, Teves ME, de Oliveira GS, Garcia AA, Barratt CL, Harper CV. Ca^{2+} signalling in the control of motility and guidance in mammalian sperm. *Front Biosci* 2008: **13;** 5623-5637.

Publicover SJ, Barratt CL. Sperm motility: things are moving in the lab! *Mol Hum Reprod* 2011: **17**; 453-456.

Punt-van der Zalm JP, Hendriks JC, Westphal JR, Kremer JA, Teerenstra S, Wetzels AM. Toxicity testing of human assisted reproduction devices using the mouse embryo assay. *Reprod Biomed Online* 2009: **18;** 529-535.

Purcell EM. Life at low Reynolds number. *Am J Phys* 1977: **45**; 3-11.

Qi H, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, Kirichok Y, Ramsey IS, Quill TA, Clapham DE. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proc Natl Acad Sci USA* 2007: **104;** 1219-1223.

Raoof NT, Pearson RM, Turner P. Lithium inhibits human sperm motility in vitro. *Br J Clin Pharmacol* 1989: **28;** 715-717.

Ramadan WM, Kashir J, Jones C, Coward K. Oocyte activation and phospholipase C zeta (PLCζ): diagnostic and therapeutic implications for assisted reproductive technology. *Cell Commun Signal* 2012: **10;** 12.

Randall GW, Gantt PA. Preimplantation murine embryos are more resistant than human embryos to bacterial endotoxins.J In Vitro Fert Embryo Transf 1990: 7; 280-282.

Ravnik SE, Albers JJ, Muller CH. Stimulation of human sperm capacitation by purified lipid transfer protein. *J Exp Zool* 1995: **272**; 78-83.

Rhomberg LR. Goodman JE. Low-dose effects and nonmonotonic dose-responses of endocrine disrupting chemicals: Has the case been made? *Regul Toxicol Pharmacol* 2012: **64;** 130-133.

Richardson DW, Sadleir RM. The toxicity of various non-electrolytes to human spermatozoa and their protective effects during freezing. *J Reprod Fertil* 1967: **14;** 439-444.

Rogers BJ, Perreault SD, Bentwood BJ, McCarville C, Hale RW, Soderdahl DW. Variability in the human-hamster in vitro assay for fertility evaluation. *Fertil Steril* 1983: **39**; 204-11.

Ross C, Morriss A, Khairy M, Khalaf Y, Braude P, Coomarasamy A, El-Toukhy T. A systematic review of the effect of oral antioxidants on male infertility. *Reprod Biomed Online* 2010: **20**; 711-723.

Rossato M, Balercia G, Lucarelli G, Foresta C, Mantero F. Role of seminal osmolarity in the regulation of human sperm motility. *Int J Androl* 2002: **25**; 230-235.

Rowley MJ, Leach DR, Warner GA, Heller CG. Effect of graded doses of ionising radiation on the human testis. *Radiat Res* 1974: **59**; 665-678.

Roy Choudhury D, Small C, Wang Y, Mueller PR, Rebel VI, Griswold MD, McCarrey JR. Microarray-based analysis of cell-cycle gene expression during spermatogenesis in the mouse. *Biol Reprod* 2010: **83;** 663-675.

Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 2010: **93;** 1027-1036.

Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner I, Jaquenoud N, Manicardi G, Campana A. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum Reprod* 1996: **11;** 837-843.

Saleh RA, Agarwal A, Kandirali E, Sharma RK, Thomas AJ, Nada EA, Evenson DP, Alvarez JG. Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fert Steril* 2002: **78**; 1215-1224.

Salicioni AM, Platt MD, Wertheimer EV, Arcelay E, Allaire A, Sosnik J, Visconti PE. Signalling pathways involved in sperm capacitation. *Soc Reprod Fertil Suppl* 2007: **65**; 245-259.

Sánchez-Pozo MC, Mendiola J, Serrano M, Mozas J, Björndahl L, Menkveld R, Lewis SE, Mortimer D, Jørgensen N, Barratt CL, Fernández MF, Castilla JA; on behalf of the Special Interest Group in Andrology (SIGA) of the European Society of Human Reproduction and Embryology. Proposal of guidelines for the appraisal of SEMen QUAlity studies (SEMQUA). *Hum Reprod* 2012: doi: 10.1093/humrep/des355. [Epub ahead of print]

Sathananthan AH. Human centriole: origin, & how it impacts fertilization, embryogenesis, infertility & cloning. *Indian J Med Res* 2009: **129;** 348-350.

Sathananthan AH, Ratnam SS, Ng SC, Tarín JJ, Gianaroli L, Trounson A. The sperm centriole: its inheritance, replication and perpetuation in early human embryos. *Hum Reprod* 1996: **11;** 345-356.

Sathananthan AH, Selvaraj K, Girijashankar ML, Ganesh V, Selvaraj P, Trounson AO. From oogonia to mature oocytes: inactivation of the maternal centrosome in humans. *Microsc Res Tech* 2006: **69;** 396-407.

Schalet SM. Effects of cancer chemotherapy on gonadal function in the human. *Cancer Treatment Reviews* 1980: **7**; 141-152.

Schatten G. The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. *Dev Biol* 1994: **165;** 299-235.

Scheike TH, Rylander L, Carstensen L, Keiding N, Jensen TK, Stromberg U, Joffe M, Akre O. Time trends in human fecundability in Sweden. *Epidemiology* 2008: **19**; 191-196.

Schill WB, Comhaire FH, Hargreave TB (eds). *Andrology for the Clinician.* 2006. Springer-Verlag, Berlin, Germany.

Schrader SM. Male reproductive toxicity. In Massaro EJ (ed) Handbook of Human Toxicology. 1997. CRC Press, Boca Raton, USA, pp. 962-980.

Schrader SM, Turner TW, Simon SD. Longitudinal study of semen quality of unexposed workers. Sperm motility characteristics. *J Androl* 1991: **12**; 126-131.

Schröter S, Osterhoff C, McArdle W, Ivell R. The glycocalyx of the sperm surface. *Hum Reprod Update* 1999: **5;** 302-313.

Schütte B. Penetration ability of human spermatozoa into standardized bovine cervical mucus (Penetrak) in patients with normal and pathological semen samples. *Andrologia* 1987: **19 Spec No**; 217-224.

Sedat J, Agard D, Shuman M, Stroud R. Letter of concern. www.npr.org/assets/news/2010/05/17/concern.pdf, 2010; accessed 22 October 2012.

Seibert H, Kolossa M, Wassermann O. Bovine spermatozoa as an in vitro model for studies on the cytotoxicity of chemicals: effects of chlorophenols. *Cell Biol Toxicol* 1989: **5;** 315-330.

Seli E, Sakkas D. Spermatozoal nuclear determinants of reproductive outcome: implications for ART. *Hum Reprod Update* 2005: **11;** 337-349.

Serres C, Escalier D, David G. Ultrastructural morphometry of the human sperm flagellum with a stereological analysis of the lengths of the dense fibres. *Biol Cell* 1983: **149**; 153-162.

Serres C, Feneux D, David G. Microcinematographic analysis of the motility of human spermatozoa incubated with caffeine. *Andrologia* 1982: **14**; 454-460.

Shalgi R, Smith TT, Yanagimachi R. A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through

the uterotubal junction. *Biol Reprod* 1992; **46**: 419-424.

Sharpe RM. Pathways of endocrine disruption during male sexual differentiation and masculinisation. *Best Pract Res Clin Endocrinol Metab* 2006: **20**; 91-110.

Sharpe RM. Male reproductive health disorders and the potential role of exposure to environmental chemicals. 2009. CHEM Trust, Bishops Lydeard, UK. www.chemtrust.org.uk/documents/ProfRSHARPE-MaleReproductiveHealth-CHEMTrust09.pdf, accessed 22 October 2012.

Sharpe RM. Environmental/lifestyle effects on spermatogenesis. *Phil Trans R Soc Lond B Biol Sci* 2010: **365;** 1697-1712.

Sharpe RM. Sperm counts and fertility in men: a rocky road ahead. Science & Society Series on Sex and Science. *EMBO Rep* 2012: **13;** 398-403.

Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm count and disorders of the male reproductive tract? *Lancet* 1993: **341**; 1392-1395.

Sharpe RM, Skakkebaek NE. Male reproductive disorders and the role of endocrine disruption: Advances in understanding and identification of areas for future research. *Pure Appl Chem* 2003: **75**; 2023-2038.

Sheiner EK, Sheiner E, Hammel RD, Potashnik G, Carel R. Effect of occupational exposures on male fertility: Literature review. *Industrial Health* 2003: **41**; 55-62.

Sheng ZG, Zhu BZ. Low concentrations of bisphenol A induce mouse spermatogonial cell proliferation by G protein-coupled receptor 30 and estrogen receptor-α? *Environ Health Perspect* 2011: **119;** 1775-1780.

Sheynkin Y, Welliver R, Winer A, Hajimirzaee F, Ahn H, Lee K. Protection from scrotal hyperthermia in laptop computer users. *Fertil Steril* 2011: **95**; 647-651.

Shimonovitz S, Yagel S, Zacut D, Ben Chetrit A, Ever-Hadani P, Har-Nir R, Ron M. Ultrasound transmission gel in the vagina can impair sperm motility. *Hum Reprod* 1994: **9**; 482-483.

Showell MG, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. Antioxidants for male subfertility. *Cochrane Database Syst Rev* 2011: **19**; CD007411.

Schrader SM, Chapin RE, Clegg ED, Davis RO, Fourcroy JL, Katz DF, Rothmann SA, Toth G, Turner TW, Zinaman M. Laboratory methods for assessing human semen in epidemiologic studies: a consensus report. *Reprod Toxicol* 1992: **6;** 275-279.

Sieme H, Harrison RA, Petrunkina AM. Cryobiological determinants of frozen semen quality, with special reference to stallion. *Anim Reprod Sci* 2008: **107;** 276-292.

Sikka SC, Champion HC, Bivalacqua TJ, Estrada LS, Wang R, Rajasekaran M, Aggarwal BB, Hellstrom WJ. Role of genitourinary inflammation in infertility: synergistic effect of lipopolysaccharide and interferon-gamma on human spermatozoa. *Int J Androl* 2001: **24**; 136-141.

Silva E, Kabil A, Kortenkamp A. Cross-talk between non-genomic and genomic signalling pathways – distinct effect profiles of environmental estrogens. *Toxicol Appl Pharmacol* 2010: **245;** 160-170.

Simon L, Carrell DT. Sperm DNA damage measured by Comet assay. In Carrell DT and Aston KI (eds) Spermatogenesis: Methods and Protocols. *Methods in Molecular Biology* 2013: **927;** 137-146.

Simon L, Lutton D, McManus J, Lewis SE. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertil Steril* 2011: **95**; 652-657.

Singh NP, Muller CH, Berger RE. Effects of age on DNA double strand breaks and apoptosis in human sperm. *Fertil Steril* 2003: 80; 1420-1430.

Skakkebaek NE, Rajpert-De-Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* 2001: **16**; 972-978.

Skakkebaek NE, Toppari J, Söder O, Gordon M, Divall S, Draznin M. The exposure of fetuses and children to endocrine disrupting chemicals: A European Society for Paediatric Endocrinology (ESPE) and Pediatric Endocrine Society (PES) call to action statement. *J Clin Endocrinol Metab* 2011: **96;** 3056-3058.

Slama R, Eustache F, Ducot B, Jensen TK, Jørgensen N, Horte A, Irvine S, Suominen J, Andersen AG, Auger J et al. Time to pregnancy and semen parameters: a cross-sectional study among fertile couples from four European cities. *Hum Reprod* 2002: **17;** 503-515.

Slama R, Kold-Jensen T, Scheike T, Ducot B, Spira A, Keiding N. How would a decline in sperm concentration over time influence the probability of pregnancy? *Epidemiology* 2004: **15;** 458-465.

Smith CG, Asche RH. Drug abuse and reproduction. *Fertil Steril* 1987: **48**; 355-373.

Smith DJ, Gaffney EA, Gadêlha H, Kapur N, Kirkman-Brown JC. Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity. *Cell Motil Cytoskeleton* 2009: **66**; 220-236.

Soares SR, Melo MA. Cigarette smoking and reproductive function. *Curr Opin Obstet Gynecol* 2008: **20;** 281-291.

Stokes-Riner A, Thurston SW, Brazil C, Guzick D, Liu F, Overstreet JW, Wang C, Sparks A, Redmon JB, Swan SH. One semen sample or 2? Insights from a study of fertile men. *J Androl* 2007: **28;** 638-643.

Strawford A, Barbieri T, Neese R, Van Loan M, Christiansen M, Hoh R, Sathyan G, Skowronski R, King J, Hellerstein M. Effects of nandrolone decanoate therapy in borderline hypogonadal men with HIV-associated weight loss. *J Acquir Immune Defic Syndr Hum Retrovirol* 1999: **20**; 137-146.

Strünker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. *Nature* 2011: **471;** 382-386.

Su TW, Xue L, Ozcan A. High-throughput lensfree 3D tracking of human sperms reveals rare statistics of helical trajectories. *Proc Natl Acad Sci USA* 2012: **109;** 16018-16022.

Suarez SS. Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. *Reprod Fertil Dev* 2007: **19;** 103-110.

Suarez SS. Control of hyperactivation in sperm. *Hum Reprod Update* 2008: **14**; 647-57.

Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. *Hum Reprod Update* 2006: **12**; 23-37.

Sutovsky P, Schatten G. Paternal contributions to the mammalian zygote: fertilization after sperm-egg fusion. *Int Rev Cytol* 2000: **195;** 1-65.

Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. *Biol Reprod* 2000: **63**; 582-590.

Sutton P, Woodruff TJ, Perron J, Stotland N, Conry JA, Miller MD, Giudice LC. Toxic environmental chemicals: the role of reproductive health professionals in preventing harmful exposures. *Am J Obstet Gynecol* 2012: **207;** 164-173.

Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod Update* 2012: **18**; 333-339.

Swann K, Yu Y. The dynamics of calcium oscillations that activate mammalian eggs. *Int J Dev Biol* 2008: **52;** 585-594.

Tas S, Lauwerys R, Lison D. Occupational hazards for the male reproductive system. *Crit Rev Toxicol* 1996: **26**; 261-307.

Tateno H, Kamiguchi Y. Evaluation of chromosomal risk following intracytoplasmic sperm injection in the mouse. *Biol Reprod* 2007: **77**; 336-342.

Taylor K, Roberts P, Sanders K, Burton P. Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. *Reprod Biomed Online* 2009: **18**; 184-189.

Teede H, Deeks A, Moran L. Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on helath across the lifespan. *BMC Med* 2010: **8;** 41-51.

Templeton AA, Mortimer D. The development of a clinical test of sperm migration to the site of fertilization. *Fertil Steril* 1982: **37**; 410-415.

Tesarik J. Appropriate timing of the acrosome reaction is a major requirement for the fertilizing spermatozoon. *Hum Reprod* 1989: **4**; 957-961.

Tesarik J. Paternal effects on cell division in the human preimplantation embryo. *Reprod Biomed Online* 2005: **10;** 370-375.

Tesh JM, Glover TD. Ageing of rabbit spermatozoa in the male tract and its effect on fertility. *J Reprod Fertil* 1969: **20**; 287-297.

Tingen C, Stanford JB, Dunson DB. Methodologic and statistical approaches to studying human fertility and environmental exposure. *Environ Health Perspect* 2004: **112;** 87-93.

Tollner TL, Venners SA, Hollox EJ, Yudin AI, Liu X, Tang G, Xing H, Kays RJ, Lau T, Overstreet JW, Xu X, Bevins CL, Cherr GN. A common mutation in the defensin DEFB126 causes impaired sperm function and subfertility. *Sci Transl Med* 2011: **3**; 92ra65.

Tomlinson MJ, Pacey AA. Practical aspects of sperm banking for cancer patients. *Hum Fertil* 2003: **6**; 100-105.

Toovey S, Hudson E, Hendry WF, Levi AJ. Sulphasalazine and male infertility: reversibility and possible mechanism. *Gut* 1981: **22;** 445-451.

Toppari J, Larsen J, Christiansen P, Giwercman A, Grandjean P, Guillette Jr. LJ, Jégou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Müller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE. Male Reproductive Health and Environmental Xenoestrogens. *Environ Health Perspec* 1996: **104(Suppl 4)**; 741-803.

Torre DL, Falorni A. Pharmacological causes of hyperprolactinemia. *Ther Clin Risk Manag* 2007: **3**; 929-951.

Torres ER, Abad C, Piñero S, Proverbio T, Marín R, Proverbio F, Camejo MI. Effect of ultraviolet C irradiation on human sperm motility and lipideroxidation. *Int J Radiat Biol* 2010: **86;** 187-193.

Tournaye H, Devroey P, Camus M, Van der Linden M, Janssens R, Van Steirteghem A. Use of pentoxifylline in assisted reproductive technology. *Hum Reprod* 1995: **10 Suppl 1**; 72-79.

Tournaye H, Janssens R, Devroey P, van Steirteghem A. The influence of pentoxifylline on motility and viability of spermatozoa from normozoospermic semen samples. *Int J Androl* 1994a: **17**; 1-8.

Tournaye H, Van der Linden M, Van den Abbeel E, Devroey P, Van Steirteghem A. The effect of pentoxifylline on mouse in-vitro fertilization and early embryonic development. *Hum Reprod* 1994b: **9**; 1903-1908.

Traub AI, Earnshaw JC, Brannigan PD, Thompson W. A critical assessment of the response to caffeine of human sperm motility. *Fertil Steril* 1982: **37**; 436-437.

Tulandi T, McInnes RA. Vaginal lubricants: effect of glycerin and egg white on sperm motility and progression in vitro. *Fertil Steril* 1984: **41**; 151-153.

Turner RM. Moving to the beat: a review of mammalian sperm motility regulation. *Reprod Fertil* Dev 2006: **18**; 25-38.

Urata K, Narahara H, Tanaka Y, Egashira T, Takayama F, Miyakawa I. Effect of endotoxin-induced reactive oxygen species on sperm motility. *Fertil Steril* 2001: **76**; 163-166.

Uygur MC, Gür E, Arik AI, Altug U, Erol D. Erectile dysfunction following treatments of benign prostatic hyperplasia: a prospective study. *Andrologia* 1998: **30**;5-10.

van Ahlen H, Kliesch S. Disorders of Erection, Cohabitation, and Ejaculation. In Nieschlag E, Behre HM, Nieschlag S (eds). *Andrology: Male Reproductive Health and Dysfunction*, 3rd edition. 2010. Springer-Verlag, Berlin, Germany, pp. 279-321.

Van Waeleghem K, De Clercq N, Vermeulen L, Schoonjans F, Comhaire F. Deterioration of sperm quality in young healthy Belgian men. *Hum Reprod* 1996: **11**; 325-329.

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). *Reprod Toxicol* 2007: **24;** 139-177.

Vandeweghe M, Vermeulin L, Comhaire F. Adverse effect of caffeine on fertilizing capacity of cryopreserved humans sperm? In Spira A and

Jouannet P (eds) Human Fertility Factors. *Editions INSERM, Paris* 1982: **103**; 515-516.

Vargas J, Crausaz M, Senn A, Germond M. Sperm toxicity of "nonspermicidal" lubricant and ultrasound gels used in reproductive medicine. *Fertil Steril* 2011: **95**; 835-836.

Vassena R, Boué S, González-Roca E, Aran B, Auer H, Veiga A, Izpisua Belmonte JC. Waves of early transcriptional activation and pluripotency program initiation during human preimplantation development. *Development* 2011: **138**; 3699-709.

Velázquez A, Pedrón N, Delgado NM, Rosado A. Osmolality and conductance of normal and abnormal human seminal plasma. *Int J Fertil* 1977: **22**; 92-97.

Visconti PE. Understanding the molecular basis of sperm capacitation through kinase design. *Proc Natl Acad Sci USA* 2009: **106**; 667-668.

Visconti PE, Florman HM. Mechanisms of sperm-egg interactions: between sugars and broken bonds. *Sci Signal* 2010: **3(142)**; pe35.

Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, Kopf GS. Cholesterol efflux-mediated signal transduction in mammalian sperm. ß-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *J Biol Chem* 1999: **274**; 3235-3242.

Visconti PE, Krapf D, de la Vega-Beltrán JL, Acevedo JJ, Darszon A. Ion channels, phosphorylation and mammalian sperm capacitation. *Asian J Androl* 2011: **13**; 395-405.

Waite E, Kershaw Y, Spiga F, Lightman SL. A glucocorticoid sensitive biphasic rhythm of testosterone secretion. *J Neuroendocrinol* 2009: **21**; 737-741.

Wang G, Shao SH, Weng CC, Wei C, Meistrich M. Hormonal suppression restores fertility in irradiated mice from both endogenous and donor derived stem spermatogonia. *Toxicol Sci* 2010: **117**; 225-237.

Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 2000: **60-61;** 481-492.

Welsh M, Saunders PTK, Fisken M, Scott HM, Hutchison GR, Smith LB, Sharpe RM. Identification in rats of a programming window for reproductive tract masculization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* 2008: **118;** 1479-1490.

Weng SP, Wu TC, Chen SU, Wu J, Lin CC, Yang YC, Chen PC. The impact of ethylene glycol monomethyl ether on ovarian function may extend to the next generation in female mice: a preliminary study. *Reprod Toxicol* 2010: **29**; 452-457.

Whan LB, West MC, McClure N, Lewis SE. Effects of delta-9-tetrahydrocannabinol, the primary psychoactive cannabinoid in marijuana, on human sperm function in vitro. *Fertil Steril* 2006: **85;** 653-660.

White IG. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod Fertil Dev* 1993: **5;** 639-658.

Williams M, Hill CJ, Scudamore I, Dunphy B, Cooke ID, Barratt CL. Sperm numbers and distribution within the human fallopian tube around ovulation. *Hum Reprod* 1993: **8**; 2019-2026.

Wirth JJ, Mijal RS. Adverse effects of low level heavy metal exposure on male reproductive function. *Syst Biol Reprod Med* 2010: **56**; 147-167.
Withers HR, Mason KA, Davis CA. MR effect on murine spermatogenesis. *Radiology* 1985: **156**; 741-742.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Reijo Pera RA. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010: **28**; 1115-1121.

Woodruff TJ. Bridging epidemiology and model organisms to increase understanding of endocrine disrupting chemicals and human health effects. *J Steroid Biochem Mol Biol* 2011: **127;** 108-117.

Woodruff TJ, Carlson A, Schwartz JM, Guidice LC. Proceedings of the Summit on Environmental Challenges to Reproductive Health and Fertility: Executive summary. *Fertil Steril* 2008: **89**; 281-300.

Woolley DM. Interpretations of the pattern of sperm tail movements. In Fawcett DW and Bedford JM (eds) The Spermatozoon. 1979. Urban & Schwarzenberg, Baltimore, USA, pp. 69-79.

Woolley DM. Flagellar oscillation: a commentary on proposed mechanisms. *Biol Rev Camb Philos Soc* 2010: **85;** 453-470.

World Health Organization. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 3rd edition. 1992. Cambridge University Press, Cambridge, UK.

World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 2010. World Health Organization, Geneva, Switzerland.

Wyrobek AJ, Gordon LA, Burkhart JG, Francis MW, Kapp RW Jr, Letz G, Malling HV, Topham JC, Whorton MD. An evaluation of the mouse sperm morphology test and other sperm tests in nonhuman mammals. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res* 1983a: **115;** 1-72.

Wyrobek AJ, Gordon LA, Burkhart JG, Francis MW, Kapp RW Jr, Letz G, Malling HV, Topham JC, Whorton MD. An evaluation of human sperm as indicators of chemically induced alterations of spermatogenic function. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res* 1983b: **115;** 73-148.

Wyrobek AJ, Schrader SM, Perreault SD, Fenster L, Huszar G, Katz DF, Osorio AM, Sublet V, Evenson D. Assessment of reproductive disorders and birth defects in communities near hazardous chemical sites. III: Guidelines for field studies of male reproductive disorders. *Reprod Toxicol* 1997: **11**; 243-259.

Xu B, Wang ZP, Wang YJ, Lu PH, Wang LJ, Wang XH. The toxic effect of opioid analgesics on human sperm motility in vitro. *Drug Chem Toxicol* 2012 doi:10.3109/01480545.2012.710621 Epub ahead of print.

Yanagimachi R. The movement of golden hamster spermatozoa before and after capacitation. *J Reprod Fertil* 1970: **23**; 193-196.

Yanagimachi R. Mammalian fertilization. In Knobil E and Neill JD (eds) The Physiology of Reproduction, 2nd edition. 1994. Raven Press, New York, pp. 189-317.

Yanagimachi R. Mammalian sperm acrosome reaction: Where does it begin before fertilization? *Biol Reprod* 2011: **85**; 4-5.

Yeung CH, Callies C, Tüttelmann F, Kliesch S, Cooper TG. Aquaporins in the human testis and spermatozoa - identification, involvement in sperm volume regulation and clinical relevance. *Int J Androl* 2010: **33**; 629-641.

Yiee JH, Baskin LS. Environmental factors in genitourinary development. *J Urol* 2010: **180;** 34-41.

Yoshida M, Yoshida K. Sperm chemotaxis and regulation of flagellar movement by Ca^{2+}. *Mol Hum Reprod* 2011: **17;** 457-465.

Yunes R, Doncel GF, Acosta AA. Incidence of sperm-tail tyrosine phosphorylation and hyperactivated motility in normozoospermic and asthenozoospermic human sperm samples. *Biocell* 2003: **27;** 29-36.

Zanetti N, Mayorga LS. Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. *Biol Reprod* 2009: **81**; 396-405.

Zargooshi J. Sperm count and sperm motility in incidental high-grade varicocele. *Fertil Steril* 2007: **88**; 1470-1473.

Zhang X, San Gabriel M, Zini A. Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. *J Androl* 2006: **27**; 414-420.

Zhu QX, Huang YF. Impact of endocrine disrupting chemicals on semen quality. *National J Androl* 2011: **17;** 943-946. Article in Chinese: *Zhonghua Nan Ke Xue* 2011: **17;** 943-946.

Zinaman M, Drobnis EZ, Morales P, Brazil C, Kiel M, Cross NL, Hanson FW, Overstreet JW. The physiology of sperm recovered from the human cervix: acrosomal status and response to inducers of the acrosome reaction. *Biol Reprod* 1989: **41;** 790-797.

Zini A, Al-Hathal N. Antioxidant therapy in male infertility: fact or fiction? *Asian J Androl* 2011: **13**; 374-381.

Zini A, San Gabriel M, Baazeem A. Antioxidants and sperm DNA damage: a clinical perspective. *J Assist Reprod Genet* 2009: **26**; 427-432.

Zorgniotti AW. Elevated intrascrotal temperature. I: A hypothesis for poor semen in infertile men. *Bull NY Acad Med* 1982: **58;** 535-540.

Zorgniotti AW, Sealfon AI. Scrotal hypothermia: new therapy for poor semen. *Urology* 1984: **23;** 439-441.

Zorgniotti A, Reiss H, Toth A, Sealfon A. Effect of clothing on scrotal temperature in normal men and patients with poor semen. *Urology* 1982: **19;** 176-178.

Zorgniotti AW, Cohen MS, Sealfon AI. Chronic scrotal hypothermia: results in 90 infertile couples. *J Urol* 1986: **135;** 944-947.

Table 1: Major environmental contaminants, their sources and selected health effects from developmental and adult exposure (combined animal and human data). Broad categories underlined; effects on semen or sperm quality shown in bold. Adapted from Woodruff et al. (2008) and expanded.

Contaminant	Specifics	Sources	Reported health effects associated with exposure	
			during development	during adulthood
Bisphenol A	Industrial chemical and component of polycarbonate plastic and epoxy resins.	Lining of metal food and drink cans; hard plastic water bottles; plastic baby bottles, pacifiers and baby toys; dental sealants; computers, cell phones, CDs and DVDs; paints, adhesives, enamels, and varnishes; some microwavable or reusable food and drink containers.	Altered puberty onset; hormonal changes; altered prostate development; **decreased semen quality**; obesity.	**Decreased semen quality**; oocyte chromosome abnormalities; recurrent miscarriage.
<u>Chlorinated hydrocarbons</u>				
Dioxins / Furans	Byproducts of the manufacture and burning of products that contain chlorine.		Reproductive tract malformations.	Menstrual irregularities. Transgenerational epigenetic reproductive disorders
Organochlorine pesticides	Largely banned in the US. Persist for decades in the environment. Accumulate up the food chain.	Examples include DDT (dichloro-diphenyltrichloroethane), Chlordane, HCB (hexachlorobenzene).	Altered sex ratio; altered puberty onset; **decreased semen quality**; delayed time to pregnancy.	Altered puberty onset; **decreased semen quality**. Altered menarche onset; endometriosis; fetal loss.
Pentachloro-phenol	Wood preservative for utility poles, railroad ties, wharf pilings. Formerly used as a pesticide.			
Polychlorinated biphenols (PCBs)	Banned in the US in 1976. Persist for decades in the environment. Accumulate up the food chain.	Industrial insulators and lubricants.	Reduced fertility; altered estrous cycle.	Hormonal changes; reduced fertility.
Disinfection by-products	Over 600 compounds formed by the reaction of chemical disinfectants (most often chlorine) with natural organic matter, primarily in surface waters. Most prevalent = trihalomethanes.		Fetal growth retardation	Menstrual irregularities; **sperm, oocyte and embryo toxicity**.
Ethylene oxide	Chemical sterilizer used in dental and medical practices and for some medical devices.			**Decreased semen quality**; miscarriage / fetal loss.
Glycol ethers	Used in many formulated products.	Paints, enamels, varnishes, thinners and wood stains; printing inks; electronics and semi-conductor industry; leather; cosmetics; perfumes.		Reduced fertility; **decreased semen quality**; longer menstrual cycles; fetal loss.

Nonylphenol Octylphenol	Primary exposure is from drinking water contaminated by sewage and wet-weather runoff.	Used to make surfactants (detergents); pesticides; paints; plasticizers and UV stabilizers in plastics; and in many other formulated products.	Hormonal changes; altered puberty onset; **decreased testicular size; decreased semen quality**.	
Perfluorinated compounds (PFOS, PFOA)	Accumulate in the environment and the food chain.	Water-repellant treatments for fabrics & carpets; non-stick coatings for cooking pans; floor polish; insecticides; food wraps.	Hormonal changes; reduced birth weight; fetal loss.	
<u>Metals</u>				
Cadmium	Used in industry and consumer products, mainly batteries, pigments, metal coatings, plastics, and some metal alloys.		**Damage to Sertoli cells, testicular development.**	**Damage to Sertoli cells; highly damaging to spermatogenesis.**
Lead	Used in batteries, ammunition, metal products, X-ray shields. Reduced use as a gasoline additive and in paints, ceramic products, caulking, and solder.	Most common source of exposure in first world countries is lead-based paint in older homes, lead-contaminated house dust and soil and vinyl products.	Hormonal changes; altered puberty onset.	Hormonal changes; menstrual irregularities; reduced fertility; fetal loss. **Altered puberty onset; reduced spermatogenesis; abnormal spermatozoa.**
Manganese	Used in the production of batteries, in dietary supplements, and as ingredients in some ceramics, pesticides, and fertilizers. Gasoline additive.			
Mercury	Air and water contaminated by industrial emissions and the combustion of coal and waste. Accumulates in food chain; most common source of exposure in US is contaminated seafood.	Used in thermometers, dental fillings, batteries, vaccines and other industries.		**Highly damaging to spermatogenesis.**
<u>Pesticides</u>	Broad category, includes many classes of insecticides, fungicides, herbicides, rodenticides, and fumigants.	Pesticides in industrial, agricultural and residential settings. Exposure can occur through food, drinking water, inhalation or absorption through the skin.	Altered sex ratio; malformations of reproductive tract; altered puberty onset; reduced fertility; retarded fetal growth.	Hormonal changes; menstrual irregularities; reduced fertility; **decreased semen quality; sperm chromosome anomalies**; miscarriage.
	See also: organochlorine pesticides, , nonylphenol, octyphenol, pentachlorophenol, perfluorinated compounds.			

Pharmaceuticals	Many examples, e.g. DES (diethylstilbestrol), ethynylestradiol (birth control pill)		Reproductive tract malformations; altered hormone response; menstrual irregularities; reduced fertility; uterine fibroids; miscarriage.	Numerous effects on the male and/or female reproductive systems. Those **affecting sperm production or quality** include anabolic steroids, calcium channel blockers; colchicine, sulphasalazine, testosterone, (see text).
Phthalates	Plasticizers added to soften plastics like PVC; also used in cosmetics and wood finishers.	Cosmetics; perfumes; toys; pharmaceuticals; medical devices; lubricants.	Malformations of reproductive tract; hormonal changes; **decreased semen quality**.	Earlier menarche; estrous cycle irregularity; reduced fertility; dysovulation; endometriosis; **decreased semen quality;** fetal loss.
Polybrominated Diphenyl Ethers (PBDEs)	Accumulate in the food chain.	Flame retardants used in furniture foam, mattresses, textiles, computers and electronics.		**Decreased semen quality.**
Solvents (organic)	Benzene, toluene, xylene, styrene, 1-bromopropane, 2-bromopropane, perchloroethylene, trichloroethylene, and many others. Solvents are some of the highest production volume chemicals. Also found in cigarette smoke.	Used in plastics, resins, rubbers, synthetic fibres, lubricants, dyes, detergents, drugs, pesticides, glues, paints & paint thinners, fingernail polish, lacquers, detergents, printing and leather tanning processes, insulation, fibreglass, food containers, carpet backing, cleaning products. Exposure is primarily through respiration.		Hormonal changes; reduced fertility; menstrual irregularities; **decreased semen quality**; miscarriage and fetal loss.
Tobacco smoke	Several hundred components.	Includes active and/or passive smoking	Decreased fetal growth; low birth weight; pre-term delivery; low birth weight; **decreased semen quality**.	Hormonal changes; reduced fertility; **decreased semen quality**; miscarriage; early menopause.

Table 2: Overview of substances and physico-chemical factors that have established direct effects on sperm function in vitro. See text for details and bibliography.

Substances	**Effects on sperm**					**Comments and established modes of action**
	Motility	**C**apacitation	**A**crosome reaction	**V**itality	**D**NA / chromatin	
Psychotropic drugs (anti-depressants, anti-epileptics, e.g. lithium, propranolol)	M					
Opioid analgesics	M					
Calcium channel blockers	M	C	A	V		Disturbed Ca^{2+} homeostasis
Phosphodiesterase inhibitors (e.g. caffeine, theophylline, pentoxifylline)	M	C	A	V		Can stimulate motility and capacitation if used at lower doses, but induce metabolic stress ("burn-out") at higher concentrations
Statins	M	C	A	V		Deplete membrane cholesterol
Cannabis (recreational drug) as THC	M		A			
Alcohols (e.g. ethanol, methanol, iso-propyl alcohol)	M			V		Membrane protein denaturation (fixation)
Tobacco smoke byproducts	M			V	D	ROS generation
Glycerol, propylene glycol	M			V	D	Membrane permeabilization (glycerol safe at low concentrations and if added slowly)
Spermotoxic products (e.g. nonoxynol-9)	M			V		Membrane permeabilization, primarily act as biocompatible detergents
Detergents and soaps	M			V		Membrane permeabilization
Calcium chelators (e.g EDTA)	M					Also likely effects on capacitation and acrosome reaction
Oils (e.g. "baby oil", mineral oil, canola)				V		Act on lipophilic membrane components, also contain pro-oxidative by-products
Lubricants / intimate mositurizers	M			V		Typically via hyperosmolarity, low/high pH, or membrane permeabilization, depending on constituents
Ultrasound coupling gels				V		Via hyperosmolarity, low/high pH, or membrane permeabilization, depending on constituents

Substances	**Effects on sperm**					**Comments and established modes of action**
Reactive oxygen species	M	C	A	V	D	
Endotoxin (pyrogens)	M			V	D	Via ROS generation
Electromagnetic radiation: UV light	M			V		Via ROS generation
Radio frequency emissions (e.g. mobile phones)	M			V	D	Via ROS generation
Temperature (e.g. “cold shock”)	M			V		
Osmolarity (outside the physiological range)	M			V		
pH (outside the physiological range)	M	C	A	V		

Table 3: Summary of the series of processes leading to conception that involve sperm production and sperm function. Dysfunction in any process will contribute to reduced fertility potential, and failure will cause sterility.

Processes		Tract	Location	Evaluation*	
Sperm production	Spermatogenesis	Male	Testis	Testis biopsy or FNA with histology	Semen analysis
	Spermiogenesis		Testis		
	Spermiation		Testis		
	Maturation		Epididymis (caput, corpus)		
	Storage		Epididymis (cauda; in man includes the "convoluted vas")	Semen analysis	
Sperm transfer	Erection	Male	Penis		
	Ejaculation		Penis + accessory glands		
	Insemination	Female	Vagina	PCT	
Gamete approximation	Penetration into cervical mucus	Female	Cervix	PCT, SMIT	
	Migration through cervical mucus		Cervix	SFT / LSR	
	Uterine transit		Uterus	LSR	
	Sperm reservoir		Oviduct isthmus	?	
	Movement to site of fertilization		Oviduct isthmus to ampulla	LSR	
Sperm capacitation		Female	Cervix to oviduct ampulla	SFT	
Sperm-oocyte interaction (fertilization)	Penetration through the cumulus oophorus	Female	Oviduct ampulla	IVF	
	Penetration through the corona radiata		Oviduct ampulla		
	Binding to the zona pellucida		Oviduct ampulla	IVF, ZBT and possible future tests based on rhZP3	
	Induction of the acrosome reaction		Oviduct ampulla	SFT	
	Penetration through the zona pellucida		Oviduct ampulla	ZBT, IVF	
	Binding to the oolemma		Oviduct ampulla	HEPT	IVF
	Incorporation into the oocyte		Oviduct ampulla	HEPT	IVF
	Male pronucleus formation		Oviduct ampulla	IVF	
	Syngamy		Oviduct ampulla		
Early embryonic development		Female	Oviduct ampulla & isthmus	IVF embryo culture	

*Abbreviations:

FNA = *fine needle aspiration.*
HEPT = *possible within the zona-free hamster egg penetration test (but rarely used).*
IVF = *can only be evaluated as part of clinical IVF treatment.*
LSR = *laparoscopic sperm recovery (Mortimer and Templeton, 1982; Templeton and Mortimer, 1982), but rarely used.*
PCT = *post-coital test*.
SFT = *in vitro sperm function testing method(s) available (see text for more detail).*
SMIT = *sperm-mucus interaction tests (in vitro), no longer performed in most centres.*
ZBT = *sperm-zona binding tests (includes hemi-zona assay), rarely performed due to lack of human zonae.*

Table 4: Current generation tests covering the spectrum of biological functions that spermatozoa must be able to perform or achieve without compromise following exposure to a substance in order for that substance to be described as "sperm safe" (see text for more details). Methods shown in italics are not generally/routinely available.

Process	Endpoint	Method	Reference
Sperm longevity	Sperm vitality (% live)	Sperm survival assay: Vital staining	Björndahl et al., 2010
	Sperm motility (% progressive)	Sperm survival assay: Visual assessment CASA	Björndahl et al., 2010
Sperm motility	Kinematics	CASA	Björndahl et al., 2010; Mortimer and Mortimer, 2013
	Ability to penetrate cervical mucus	Kremer Test	Björndahl et al., 2010
Capacitation	Sperm hyperactivation	CASA	Björndahl et al., 2010; Mortimer and Mortimer, 2013
Sperm-egg interaction	Acrosome reaction	Ionophore challenge test	Björndahl et al., 2010
	Sperm-zona pellucida binding	*Hemi-zona or competitive sperm-zona binding assays*	*Björndahl et al., 2010*
	Sperm-oocyte fusion	*Zona-free hamster egg penetration test / sperm penetration assay*	*[no recommended protocol]*
Oxidative stress	ROS generation	Chemiluminescence	Björndahl et al., 2010
	Lipid peroxidation	*Spectrophotometric assay of malondialdehyde*	*Gomez et al., 1998*
		Flow cytometry using BODIPY C11	*Aitken et al., 2007*
	DNA fragmentation	TUNEL assay	Björndahl et al., 2010
		Comet assay	Simon and Carrell, 2013
		Sperm chromatin structure assay (SCSA)	*Evenson, 2013*

Table 5: Summary of the criteria that should be employed for a product to be described as "sperm-safe". See text for details and background evidence.

Area of concern	Criteria	Evidence types
Precautionary principle	Substances cannot be approved based only on similarity to substances already considered safe.	Actions or molecular properties similar to substances with the potential to be sperm unsafe must be considered potentially damaging until proven safe.
Claims must be sound	Avoid claims of presumed safety for spermatozoa based on: • containing only "GRAS" constituents; • presumed comparability with "similar" predicate products; • false perceptions of physiology (such as might be acceptable in the lay literature); or • invalid extrapolations from testing in other species.	Reject such "evidence" during critical evaluation of claims.
Evidence of safety	Biological validity based on objective, properly designed and carried out experimental studies. Claims of being "sperm-safe" for products to which spermatozoa will be specifically exposed during the normal course of their use must be based on evidence covering all aspects of sperm function that might be affected by the product – a simple test on sperm motility alone cannot be considered adequate.	Studies published in peer-reviewed journals. Limited reliance on conference abstracts: even if published – not subject to rigorous peer-review. Should be published in a peer-reviewed journal (e.g. within 12–18 months) for continued citation unless publication has to be delayed due to a patent application.
	Comprehensive testing to exclude all possible aspects of spermotoxicity.	Sperm-specific testing on: • prototype products; • final product formulation; and • on each revised or "improved" formulation before release
	Other biocompatibility testing	E.g. for a vaginal lubricant, check for possible effects on: • vagina and cervix; • penis; and • embryo teratogenicity (if used during pregnancy it could be carried from the vagina into the uterine lumen via the portal blood supply).
General quality testing	On each manufactured batch of product prior to release.	Sterility, e.g. a sterility assurance level (SAL) of at least 10^{-3}. Endotoxin contamination low or undetectable, e.g. <0.5 EU/ml
Shelf life	Verify: • lack of toxicity; and • maintenance of all other pertinent quality criteria.	Repeat bioassay testing throughout the entire stated shelf life of the product.

Figures

Figure 1: Diagrammatic representations of **A**: the general morphology and dimensions of the human spermatozoon and **B**: the variety of abnormal forms (pleiomorphism) commonly seen in human spermatozoa even in the ejaculates of fertile men. Figures modified from Mortimer and Mortimer (2005).

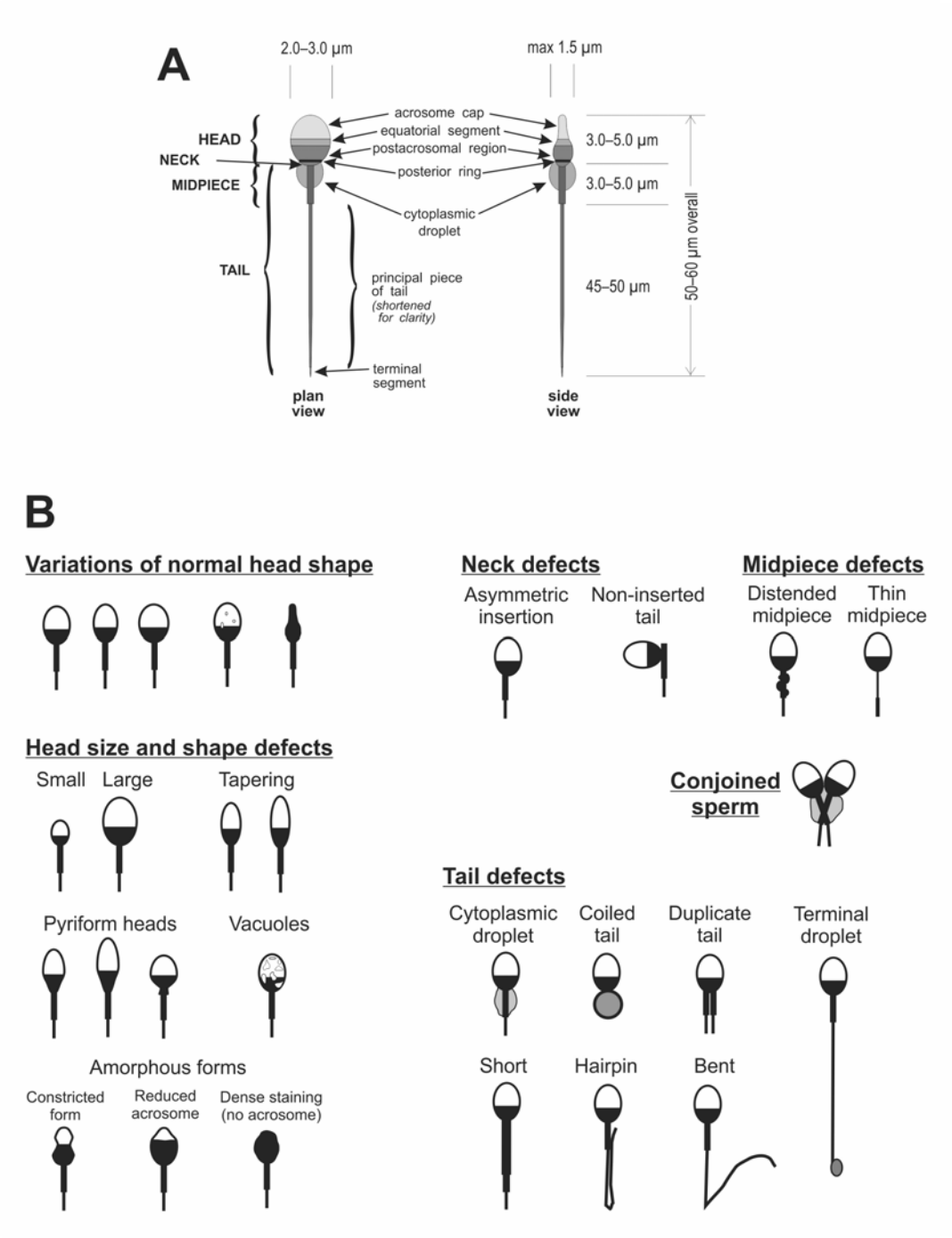

Figure 2: Fine structure of the human spermatozoon. Longitudinal sections through the sperm head parallel (**A**) and perpendicular (**B**) to the axis of the proximal centriole. Cutaway drawings show the organization of the midpiece (**C**) and principal piece (**D**) regions of the sperm tail. Reprinted with permission from Mortimer (1994), adapted from Pedersen and Fawcett (1976).

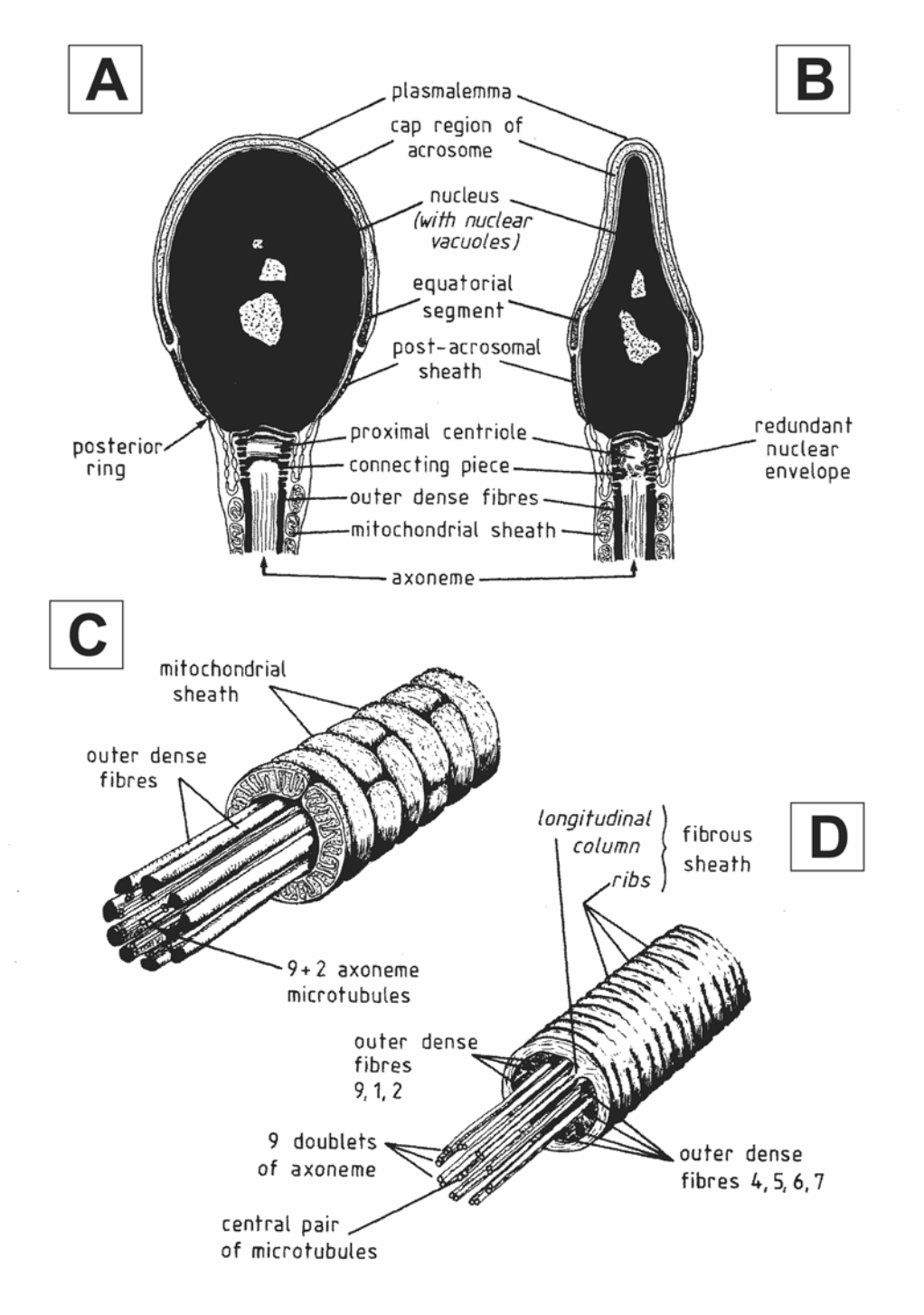

Figure 3: Sperm kinematics: (**A**) example sperm track (2-dimensional projection of the real-world 3-dimensional helical sperm trajectory) showing how the kinematic parameters describing sperm movement are derived; (**B**) example flagellar beat patterns and head centroid-derived tracks for seminal progressive and hyperactivating spermatozoa. Figure 3A modified from Mortimer and Mortimer (2005), Figure 3B based on graphics created by Dr Sharon T Mortimer.

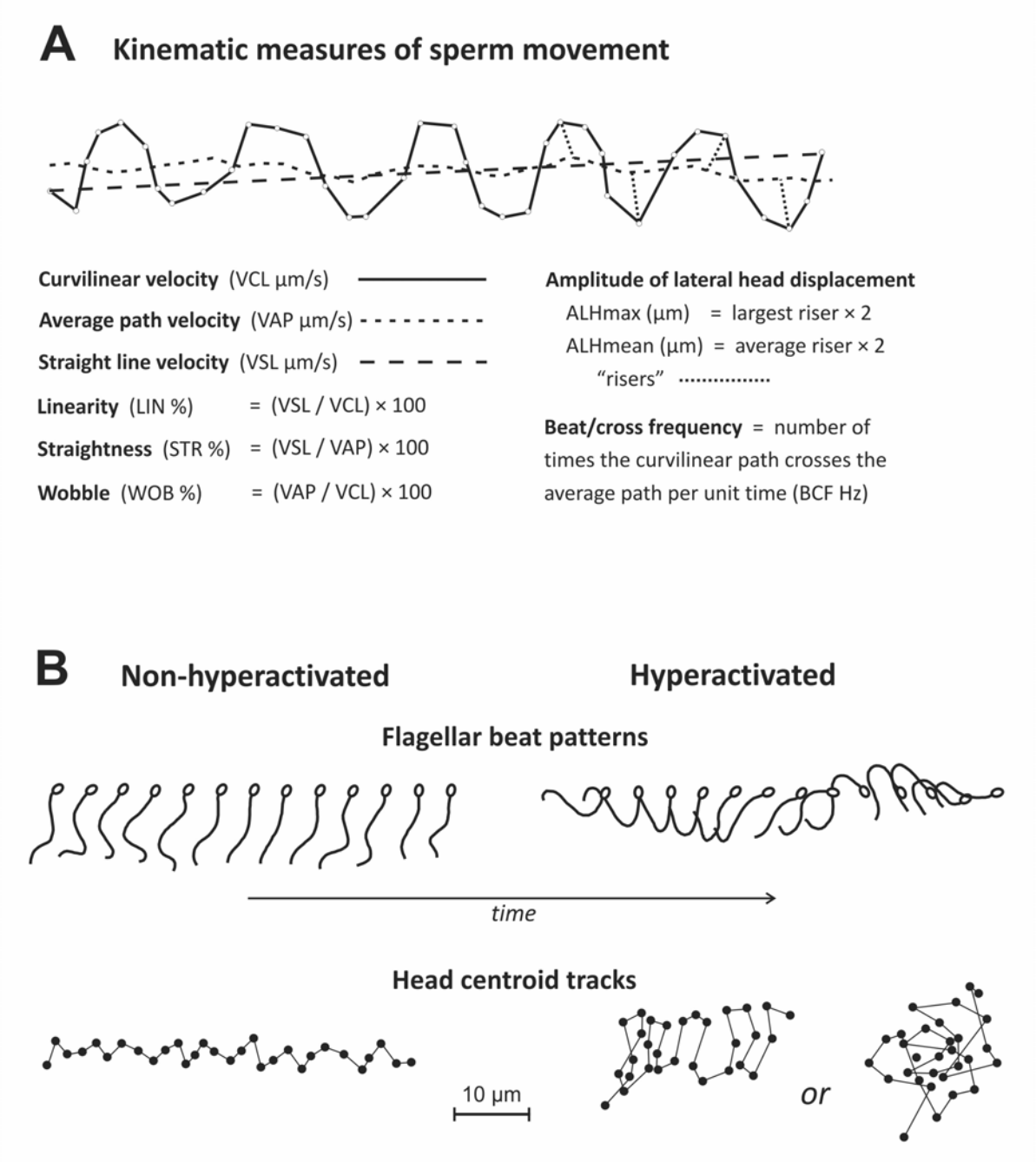

Figure 4: Flow chart illustrating a proposed pathway for developing strategies to verify that a product or substance is sperm-safe.