Human Reproduction, Vol.25, No.4 pp. 824–838, 2010
Advanced Access publication on February 6, 2010 doi:10.1093/humrep/dep465

human reproduction
POSITION REPORT *Andrology*

Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications—a position report

Christopher L.R. Barratt[1,12], R. John Aitken[2], Lars Björndahl[4], Douglas T. Carrell[5], Peter de Boer[3], Ulrik Kvist[4], Sheena E.M. Lewis[7], Sally D. Perreault[6], Melissa J. Perry[8], Liliana Ramos[3], Bernard Robaire[9], Steven Ward[10], and Armand Zini[11]

[1]Reproductive and Developmental Biology, Maternal and Child Health Science Laboratories, Centre for Oncology and Molecular Medicine, Ninewells Hospital, University of Dundee, Dundee DD1 9SY, UK [2]ARC Centre of Excellence in Biotechnology and Development and Discipline of Biological Sciences, University of Newcastle, Newcastle, NSW, Australia [3]Department of Obstetrics and Gynaecology, Radboud University Nijmegen Medical Center, P.O Box 9101, 6500 HB Nijmegen, The Netherlands [4]Centre for Andrology and Sexual Medicine, Karolinska University Hospital and Karolinska Institutet, Huddinge M52, S-141 86 Stockholm, Sweden [5]IVF and Andrology Laboratories, Departments of Surgery, Obstetrics and Gynaecology, and Physiology, University of Utah School of Medicine, 675 S. Arapeen Dr #205, Salt Lake City, UT 84108, USA [6]Office of Research and Development, U.S. Environmental Protection Agency, E205-09, 109 TW Alexander Dr., Research Triangle Park, NC 27711, USA [7]Reproductive Medicine in Centre for Public Health, Queen's University of Belfast, Institute of Clinical Science, Grosvenor Road, Belfast BT12 6BJ UK [8]Department of Environmental Health, Harvard School of Public Health, 665 Huntington Avenue, HSPH 1-1411, Boston, MA 02115, USA [9]Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montréal, QC, Canada, H3G 1Y6 [10]Institute for Biogenesis Research, University of Hawaii, 1960 East-West Rd., Honolulu, HI 96822, USA [11]Division of Urology, Department of Surgery, McGill University, St Mary's Hospital, 3830 Lacombe Avenue, Montreal, QC, Canada, H3T 1M5

[12]Correspondence address. E mail: c.barratt@dundee.ac.uk

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

This article reports the results of the most recent in a series of EHSRE workshops designed to synthesize the current state of the field in Andrology and provide recommendations for future work (for details see Appendix). Its focus is on methods for detecting sperm DNA damage and potential application of new knowledge about sperm chromatin organization, vulnerability and repair to improve the diagnosis and treatment of clinical infertility associated with that damage. Equally important is the use and reliability of these tests to identify the extent to which environmental contaminants or pharmaceutical agents may contribute to the incidence of sperm DNA damage and male fertility problems. A working group (for workshop details, see Appendix) under the auspices of ESHRE met in May 2009 to assess the current knowledgebase and suggest future basic and clinical research directions. This document presents a synthesis of the working group's understanding of the recent literature and collective discussions on the current state of knowledge of sperm chromatin structure and function during fertilization. It highlights the biological, assay and clinical uncertainties that require further research and ends with a series of 5 key recommendations.

Key words: sperm DNA damage / sperm chromatin / male infertilty / ART

Background and rationale

Traditionally, the diagnosis of male infertility is based upon microscopic assessment and analysis of sperm concentration, motility and morphology as routine indicators of human semen quality. These indicators provide fundamental information about sperm production upon which clinicians base their initial diagnosis. As emphasized in previous ESHRE reports, it is imperative that semen analysis be performed to the highest standards. To this end ESHRE's special interest group in Andrology (SIGA) has been instrumental in providing formal training programmes and external quality control schemes. However, even with appropriate quality assurance, traditional semen parameters provide a limited degree of prognostic and diagnostic information and their predictive power is highest primarily at the

© The Author 2010. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org

lower ranges of the spectrum (Lefièvre *et al*., 2007; Lewis, 2007). Sperm production is only part of the story. Sperm chromatin and DNA integrity is essential to ensure that the fertilizing sperm can support normal embryonic development of the zygote. To better inform treatment pathways and, more importantly, to ensure a generation of healthy children from assisted reproductive technologies (ART), we urgently require tests of sperm function, including the normalcy of sperm DNA, that provide high quality and robust diagnostic and prognostic information.

Improved tools for the diagnosis of male infertility clearly benefit the clinician's ability to treat an infertile couple seeking help. In addition, these tools are important in a broader social context. Infertility is no longer solely a personal problem; it has become a public health issue. In developed countries such as the European Union, birth rates have been declining at an unprecedented rate over the past half century. The extent to which birth rates may be impacted by contemporary life style factors, or by exposures to environmental contaminants or pharmaceuticals remains to be determined, but is of growing concern. Environmental epidemiology studies, which measure differences in health outcomes between groups of individuals exposed to environmental contaminants and unexposed controls, are beginning to address this question. Valid indicators of sperm quality and fertility are essential for these epidemiology studies to achieve their objectives. Equally important is the potential demographic and economic impact of the increased use of ART to treat subfertility. Depending on the country, up to 3.9% of EU births are currently from ART (Nyboe Andersen *et al*., 2009). Therefore, the use of ART may result in significant demographic and economic impacts. Part of the latest population policy strategy takes this into consideration (European Parliament, 2008; Ziebe and Devroey, 2008).

Significant progress has been made towards the development of reliable tests for sperm chromatin integrity and DNA damage. A landmark paper by Evenson *et al.* (1980) suggested that assessment of DNA integrity in sperm may be a useful and potentially independent marker of fertility for both animals and men. Clinical data followed, demonstrating higher levels of chromatin damage in men with severe semen defects (Sun *et al*., 1997) and the potential negative impact of high levels of sperm DNA damage on both natural (Evenson *et al*., 1999; Spanò *et al*., 2000) and ART conception (Larson *et al*., 2000). Subsequently, the assessment of DNA damage in the male germ line and the study of its consequences have received considerable attention. However, key questions, particularly regarding clinical significance, remain to be answered (Collins *et al*., 2008).

In animals, where DNA damage can be experimentally induced in the paternal germ line by, for example, chemotherapeutic agents, clear and strong associations have been shown between damage to the paternal genome and embryo development including effects on the new born and subsequent generations (Auroux *et al*., 1990; Hales *et al*., 1992; Fernandez-Gonzalez *et al*., 2008; Delbès *et al*., 2009). These experiments are not feasible in humans, but provide clear warnings of the potential impact of for example cancer treatments in men for the next and subsequent generations. With long-term follow-up on children born as a result of ART still in its infancy, it is essential that we are able to appreciate and translate findings from animals to humans to understand the clinical implications of using spermatozoa with highly damaged DNA in ART. Progress in this area will be dependent on fundamental improvements in our understanding of the causes and consequences of DNA damage in the male germ line and the development of robust diagnostic tests that can be incorporated into the clinical assessment of male infertility patients.

Biological uncertainties: the mysteries unfolding

The nature of the germ cell and sperm chromatin

In developing better methods for assessing clinical sperm samples for use in ART or as indicators in environmental epidemiology studies, a series of assays have been designed that evaluate the integrity of sperm DNA. These include, but are not limited to, the TUNEL assay, the sperm chromatin structure assay (SCSA), the Comet Assay and the Sperm Chromatin Dispersion Assay (for details, see section: How do we assess sperm DNA damage?) These assays are already in clinical use in some infertility centres for two primary reasons. The first is to attempt to predict future ART outcome or explain previous failure. The second is to be able to detect sperm with DNA that is sufficiently damaged as to result in transmission of genetic defects to the embryo and subsequent offspring. To optimize ART, especially ICSI, clinicians would like to be able to choose sperm with intact DNA. As conducted at present, these DNA integrity assays provide an assessment of the distribution of cells in a given ejaculate. However, these assays also destroy the cells and so cannot be used to identify or select an individual intact sperm for use in ICSI. Moreover, for most of these assays, we do not have a good understanding of what kinds of lesions they are measuring. Before they become routine clinical assays, we need to comprehend what these assays are telling us about the integrity of the male genome. This will come from a thorough knowledge of the sperm chromatin structure and how it is packaged.

Most DNA in mature human sperm is bound to protamines, as somatic histones are replaced during spermiogenesis. However, biochemical analyses of human sperm proteins indicate retention of some histones resulting in a nuclear protein composition that is about 90% protamine and 10% histone. We have a fairly clear understanding of how the protamines package human sperm DNA. The protamine-bound DNA is coiled into tightly compacted toroids that contain about 50 kb of DNA (Conwell *et al*., 2003; Hud *et al*., 1993), although the histone-bound DNA is believed to be organized into nucleosomal chromatin (Pittoggi *et al*., 1999; Zalenskaya *et al*., 2000; Wykes and Krawetz, 2003; van der Heijden *et al*., 2006; Hammoud *et al*., 2009). Sperm DNA is so well protected that, unlike somatic cell chromatin, it is resistant to nucleases (Sotolongo *et al*., 2003) and sonication (Tateno *et al*., 2000). The precise packaging of the histone portion of sperm chromatin is not well understood. We are only now beginning to appreciate how this histone bound DNA is distributed throughout the chromatin, but we still do not know the size of the histone bound DNA segments. Recent evidence suggests that histone bound DNA in sperm cells is associated with gene families that are important for cell differentiation and early embryo patterning (Hammoud *et al*., 2009).

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

How are the current assays expected to interact with sperm DNA that is associated with protamine versus histone fractions? Reagents used in the TUNEL assay, for example, would not be expected to be able to access the DNA packaged and stabilized by protamines into toroids. This is because it uses the action of the enzyme terminal deoxynucleotidyl transferase (TdT) and if protamine bound chromatin is resistant to nucleases, it would be expected to be resistant to other enzymes as well. However, improperly stabilized protamine and histone bound DNA would be expected to be accessible to most of the assays currently in use for sperm DNA assessment. TdT can access DNA breaks in nucleosomal DNA, so the TUNEL assay would be expected to reveal this type of chromatin structure. The SCSA partially denatures sperm with acid which would preferentially extract histones rather than protamines (Ballachey *et al.*, 1987; Larson-Cook *et al.*, 2003). Therefore, the SCSA would also be expected to identify single stranded DNA in histone bound sperm chromatin. However, SCSA would also measure single-stranded DNA in the protamine bound DNA if the stabilization of the protamine-DNA complex have been compromised (Dias *et al.*, 2006) Finally, most protocols for the comet assay involve high salt extraction in the presence of a reducing reagent which removes both protamines and histones (Tomsu *et al.*, 2002; McVicar *et al.*, 2004). The Comet assay therefore probably detects chromatin breaks in both types of chromatin with equal efficiency. A more detailed discussion of these points can be found elsewhere (Shaman and Ward, 2006). The questions that we need to address include (i) how can we specifically assess the DNA damage in histone bound versus protamine bound DNA in sperm chromatin (ii) how meaningful is this distinction for clinical prognosis and (iii) how can we assess the damage to the whole genome as opposed to specific fractions?

Related to these questions is our limited understanding of how the sperm chromatin is formed during spermiogenesis. We know that protamines largely replace the histones during sperm nuclear condensation and that during this process the sperm DNA is unwound by topoisomerases and other proteins through the normal and necessary induction of strand breaks (Boissonneault, 2002; Kwan *et al.*, 2003). It is clear that when this packaging is not complete, DNA single and double strand breaks appear in the fully mature sperm (Marcon and Boissonneault, 2004). Furthermore, the protamine deposition can also be incomplete, resulting in ratios of histone to protamine and of protamines 1 to protamines 2 that differ from normal. Both types of defects in spermiogenesis are associated with subfertility or infertility (Aoki *et al.*, 2006a, b; Oliva, 2006). Yet, we do not know how topoisomerase cleaves sperm DNA during spermiogenesis or how the protamines interact to form sperm chromatin toroids.

These are just some examples of the uncertainties that cloud our understanding of how abnormalities in sperm chromatin structure may be used to guide clinical decisions. A host of additional aspects of sperm DNA packaging are also emerging as potential indicators of prognosis, including chromosome position within the sperm nucleus (Zalenskaya and Zalensky, 2004), the presence of mature mRNAs (Ostermeier *et al.*, 2002, 2004) and newly described pi and microRNAs in sperm chromatin (Li *et al.*, 2001; Martins and Krawetz, 2005; Carrell, 2008). Currently, we know a lot about how sperm chromatin packaging differs from somatic cell packaging, but we do not understand the specifics of how that packaging occurs. This will be important for a real understanding of what current sperm DNA assays are measuring.

Changes in sperm chromatin during sperm transport from the testis to the oocyte: the role of zinc

Zinc is incorporated into the sperm nucleus during spermiogenesis. The chromatin contains around 8 mmol Zn^{2+}/kg which equates to one zinc ion for every 10 base pairs of the DNA, equalling one turn of the DNA-protamine helix (Kvist *et al.*, 1985). However, the role of zinc in sperm chromatin structure and function is only partly understood. The chromatin of more than 90% of human spermatozoa can be experimentally decondensed *in vitro* by exposure of freshly ejaculated sperm to the anionic detergent SDS together with the divalent cation chelating EDTA (Björndahl and Kvist, 1985; Kvist *et al.*, 1988). This observation suggests a potential rapid mechanism for decondensation of the sperm chromatin that relies on Zn^{2+} depletion and interruption of macromolecules. Thus, at ejaculation, human sperm exhibit zinc-dependent chromatin stability. However, upon *in vitro* culture, human sperm become more resistant to decondensation and subsequently require disulfide bond reduction to enable unpackaging of the chromatin. This change is enhanced when zinc is withdrawn from sperm *in vitro*, and can, to a large extent, be counteracted by storing sperm in a buffer containing Zn^{2+}. Although the role of zinc—to bind thiols—is simple, the consequences of this organization are complex based upon the following observations. (i) Zinc primarily contributes to rapidly reversible chromatin stability. (ii) If zinc is lost, it will not contribute to a sufficient stabilization of the chromatin, leaving the DNA more accessible and therefore more vulnerable to factors that might degrade it (endogenous enzymes or exogenous chemicals). (iii) Extraction of zinc can elicit chromatin decondensation if repulsion of macromolecules (DNA–protein filaments) is induced simultaneously, e.g. by phosphorylation or in experimental studies by the action of detergents like SDS (Kvist *et al.*, 1987). (iv) If macromolecules are not repelling the chromatin threads, the lack of zinc can allow the formation of disulphide bridges (S–S dependent chromatin stability).

What happens to the sperm chromatin during ejaculation, liquefaction and after liquefaction during further processing for ART? The zinc content of the sperm head and the type of chromatin stability are influenced by the composition of the surrounding ‘seminal fluid’ which comprises a mixture of various secretions that vary during ejaculation, liquefaction and after ejaculation. Normally spermatozoa are expelled in the first ejaculatory expulsions suspended in the zinc-rich prostatic fluid and the zinc-chelating seminal vesicular fluid is expulsed in later fractions. Upon mixture during and after liquefaction, spermatozoa are thus exposed to a series of differing environments that can have a marked influence on chromatin stability (Björndahl and Kvist, 2003). Of clinical importance is that in some men the emptying of prostatic fluid is delayed and sperm are expelled in primarily zinc-chelating vesicular fluid, leading to extraction of zinc from the sperm chromatin (Björndahl *et al.*, 1991). Several animal studies have reported that experimentally induced zinc deficiency affects sperm chromatin structure (Evenson *et al.*, 1980). However, studies in humans are sparse and the biological effects of a zinc depleted diet are yet to be fully explored.

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

Mechanisms of DNA damage in male germ cells and spermatozoa

One of the potential mechanisms often cited as a cause of DNA damage in the male germ line is abortive apoptosis. The general idea behind this assertion is that as male germ cells metamorphose into highly differentiated spermatozoa, they progressively loose their capacity to undergo programmed cell death in the form of apoptosis. Since these cells are transcriptionally and translationally silent, it could not be otherwise. Thus, instead of engaging in a complete apoptotic response leading to cell death, differentiating haploid germ cells are thought to undergo a restricted form of this process leading to DNA fragmentation in the nucleus whereas retaining the capacity to differentiate into mature functional spermatozoa that may still be capable of fertilization (Sakkas *et al*., 2004). Clearly, haploid germ cells are capable of activating a process that resembles apoptosis in some respects because both caspase activation and phosphatidylserine exteriorization have been observed in human spermatozoa (Weng *et al*., 2002). It is possible that by expressing apoptotic markers on their surface, senescent spermatozoa ensure that their ultimate phagocytosis in the female tract will be silent and not associated with a full-blown inflammatory response (Kurosaka *et al*., 2003).

This default senescence pathway may resemble the intrinsic apoptotic cascade in many respects but in one important detail, it is very different. In an archetypal somatic cell, stimulation of the intrinsic apoptotic pathway leads to the sudden appearance of endonucleases that are either released from the mitochondria (such as endonuclease G) or activated in the cytosol (caspase-activated deoxyribonuclease) and then they move into the nucleus to cleave the intra-nucleosomal DNA. However, in spermatozoa the physical separation of the mitochondria and cytoplasmic space from the sperm nucleus means that such mechanisms cannot be operative. As a result, the claim that 'apoptosis' is a significant cause of DNA damage in human spermatozoa might not be true for cells entering this process as mature gametes. However, sperm mitochondria represent a major source of reactive oxygen species (ROS) in these cells (Koppers *et al*., 2008), that can become activated during the intrinsic apoptotic pathway. The levels of DNA damage recorded in both unselected donors and patients attending an assisted conception clinic were highly correlated with the appearance of a marker for oxidative DNA damage, 8-hydroxy, 2′-deoxyguanosine (8OHdG; De Iuliis *et al*., 2009). Although these data could be interpreted in several different ways, one plausible explanation is that some DNA damage in the male germ line is the result of a programmed senescence pathway, characterized by the activation of mitochondrial ROS formation, oxidative DNA base damage and unresolved DNA strand breakage (Aitken and De Iuliis, 2009).

If this is the case, then a key question is not what induces spermatozoa to undergo this restricted form of apoptosis—because this is their default condition. The real question is what prevents these cells from entering this pathway. The answer to this lies in defining pro-survival factors that will prevent spermatozoa from initiating apoptosis. There are undoubtedly many potential pro-survival factors for spermatozoa in the male and female reproductive tracts; their ultimate characterization is likely to help in the development of *in vitro* culture media that will preserve the functionality and genetic integrity of gametes used for assisted conception.

The nature of the DNA damage exhibited by human spermatozoa

In the literature, most authors simply talk of generic DNA damage without reference to any particular form of molecular lesion. The ability of the TUNEL and Comet assays to detect DNA damage in the male germ line clearly means that both single and double strand DNA breaks are prominent features of these cells (Irvine *et al*., 2000; Van Kooij *et al*., 2004, Enciso *et al*., 2009). In addition, damaged sperm chromatin is known to contain base adducts. The major DNA adducts found in human sperm DNA are 8OHdG and two ethenonucleosides (1,N6-ethenoadenosine and 1,N6-ethenoguanosine). Although the former is a direct consequence of oxidative attacks on sperm DNA, the latter probably arise from exposure to 4-hydroxy-2-nonenal, a major product of lipid peroxidation (Badouard *et al*., 2008). These findings, taken in conjunction with data revealing a high correlation between DNA damage and 8OHdG expression (De Iuliis *et al*., 2009) suggest that the former is commonly the product of oxidative stress originating as a consequence of the apoptotic mechanism described above, infiltrating leukocytes, redox-cycling xenobiotics or failed antioxidant defence systems. An interesting example of the latter, albeit in animals, is the powerful antioxidant protective environment in the epididymis which when disrupted, for example by deletion of glutathione peroxidase 5, can lead to abnormal levels of DNA compaction accompanied by increased miscarriage rates (Chabory *et al*., 2009).

Another type of damage that has recently come to light is DNA cross-linking. In the tightly compacted chromatin that characterizes the mature sperm nucleus, opportunities for DNA–DNA or DNA–protein cross-linking are significantly greater than in the dispersed interphase nuclei of somatic cells. This susceptibility to cross-linking phenomena has been recognized for many years (Qiu *et al*., 1995). Recently, in an analysis of the impact of estrogens on sperm chromatin structure, catechol estrogens were shown to form dimers that then covalently cross-linked the DNA so that it became completely resistant to the decondensation protocols employed in a Comet assay including treatment with reducing agents, detergents and broad spectrum proteases (Bennetts *et al*., 2008). This susceptibility of sperm chromatin to cross-linking was subsequently confirmed using epigallocatechin gallate, the major antioxidant present in green tea (Bennetts *et al*., 2008). Importantly, severely cross-linked chromatin is commonly encountered in populations of defective human spermatozoa (Windt *et al*., 1994) although the molecular basis of such super-stabilization is still unknown. Finally two of the assays most commonly used to detect DNA damage in human spermatozoa assess the stability of sperm chromatin under severely acid (SCSA assay) or alkaline (alkaline Comet assay) assay conditions. The full nature of how these pH-exposures affect the sperm chromatin is not known with certainty at the present time.

Sperm epigenetics

One area of rapidly advancing interest is sperm epigenetics and the role that epigenetic marks in sperm chromatin may play in regulating development of the embryo (Biermann and Steger, 2007; Carrell, 2008). Epigenetics refers to methods other than DNA coding changes (polymorphisms, mutations, deletions, etc.) that can alter or regulate the expression of genes (Nanassy and Carrell, 2008;

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

Trasler, 2009). Presently, it appears that the major epigenetic regulators in sperm are DNA methylation, retention of pi and micro RNA's and binding of large portions of the genome with protamines, and post-translational modification mainly N-tail chemical modifications to retained histones of the sperm genome (Emery and Carrell, 2006). It is increasingly clear that sperm epigenetics play a role in the function of the paternal DNA following fertilization and is therefore potentially an integral component of the possible effects of DNA damage on sperm function (Hammoud *et al*., 2009).

During late spermiogenesis, 85–95% of histones are replaced in human sperm through a multi-step process (Oliva, 2006). First, the histones undergo hyperacetylation then are replaced by testes-specific variants of the histones, followed by their replacement with transition proteins. The transition proteins are then rapidly replaced by two small, basic molecules termed protamine 1 and 2 (P1, P2). P1 and P2 are normally expressed in a 1:1 ratio in human sperm, and result in a much tighter packaging of the sperm DNA, resulting in a compaction of the nucleus and cessation of gene expression (Carrell *et al*., 2008). Altered expression of protamines is associated with diminished sperm quality and reduced embryogenesis quality in couples undergoing IVF/ICSI (Aoki *et al*., 2006a, b; Oliva, 2006).

The retained histones may present another possible source of epigenetic regulation of the sperm genome. Prior studies evaluating specific regions of the genome have indicated that histone retention may not be random (Gardiner-Garden *et al*., 1998, Li *et al*., 2008). Recent studies have further evaluated the potential role of the retained histones on a genome-wide basis and shown that retained histones are not randomly distributed throughout the genome. Rather they are preferentially retained in the promoter regions of genes required during embryogenesis, micro RNAs and imprinted genes (Hammoud *et al*., 2009). Each of these classes of genes is of interest from a developmental perspective, portending a possible role in early embryogenesis. To address this, the distribution of modified variants of the histones throughout the sperm genome has been analysed. It was found that H3K4Me2 and H3K4me3, which are gene 'activating' marks, were preferentially retained in developmental genes, some of which were also bivalently marked with H3K27me3, a 'silencing' mark. This bivalent marking is similar to the bivalent 'poising' of similar genes in embryonic stem cells (Hammoud *et al*., 2009). Further analysis of the genome-wide methylation of sperm DNA demonstrated that the regions of DNA bound to activating histone modifications were generally demethylated, a further activation signal. These novel findings are striking because they indicate that sperm genes may be packaged and epigenetically modified in a manner that is necessary for poising of the genes for early embryonic expression.

The potential implications of epigenetic gene poising in sperm are numerous, including the possible effects of aberrant spermiogenesis on epigenetic marking. Presently, the data indicate that oligozoospermic males have an increased incidence of abnormal methylation of CpGs in imprinted genes (Marques *et al*., 2004, 2008; Filipponi and Feil, 2009). Recent data have shown that altered methylation is found in some, but not all, of imprinted genes in the sperm of men with abnormal protamine replacement (Hammoud *et al*., in press). The potential link between general sperm DNA damage and epigenetic alterations is not yet understood, but may be very important. Such a link may help to explain the relationship between DNA damage and normal embryonic development.

Human studies of ambient chemical exposures and sperm DNA damage

Toxicology studies expose test species to toxicants of concern and determine associated health outcomes. The toxicology literature indicates that certain environmental contaminants can, at least at experimental doses, induce sperm DNA fragmentation (Evenson and Wixon, 2005) and/or induce oxidative stress in the testes (Aitken and Roman, 2007). Although at least 70 000 chemicals are used in commerce, and at least 200 exogenous chemicals can be measured in most people at any given time, very few chemicals have been evaluated specifically for their sperm DNA damaging potential. Reproductive and multigenerational test protocols specified by federal and international agencies do not include a specific test for sperm chromatin integrity. Nevertheless, numerous epidemiologic and occupational studies have shown that exposure to at least five types of environmental chemicals, known to be ubiquitous in today's modern environment, can be associated with sperm oxidative stress or sperm DNA damage. It is important to note that epidemiology studies demonstrate associations between exposures and outcomes but do not provide definitive information about cause.

Pesticides

By their nature, pesticides and/or their metabolites are biologically active and ubiquitous in the environment due to wide scale commercial and private use. The impact of pesticides on sperm DNA damage has been evaluated in at least seven studies in the last 15 years, with four reporting positive associations (Perry, 2008). Fenvalerate exposure was associated with higher % comet tail DNA and olive tail moment (Bian *et al*., 2004) and organophosphate exposure detected as urinary metabolites in sprayers was significantly associated with DNA fragmentation index measured using SCSA (Sanchez-Pena *et al*., 2004). In the only environmental (versus occupational) exposure studies to date evaluating pesticides and DNA damage, higher levels of urinary chlorpyrifos, carbaryl and pyrethroid metabolites were associated with a higher percentage of comet tail DNA using the neutral comet assay (Meeker *et al*., 2004, 2008).

Phthalates

Phthalate esters are used in food packaging, personal care products and plastics. Therefore, environmental exposures are hard to avoid in today's world. In a sample of 379 men from subfertile couples attending an infertility clinic, urinary levels of two specific phthalate metabolites monoethyl phthalate and mono-(2-ethylhexyl) phthalate were associated with an increased percentage of comet tail DNA using the neutral comet assay (Hauser *et al*., 2007). The levels of urinary phthalates among this sample were similar to levels found in the US general population.

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs), a class of persistent organic pollutants resulting from industrial production of transformers and electrical capacitors, are highly persistent and have known endocrine disrupting properties. Although the use of PCBs was banned in the mid-1970s, environmental residues accumulate in fats and human exposure continues, largely by consumption of contaminated fish and other food. The impacts of PCBs on sperm DNA integrity using the SCSA have

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

been recently demonstrated in epidemiologic studies of European and Inuit populations (Spano *et al*., 2005; Long *et al*., 2007) and in Swedish fishermen (Rignell-Hydbom *et al*., 2005).

Metals

Metals are common in industrial processing and are distributed widely in air, water and soil. Two epidemiologic studies have suggested that non-essential metals can cause sperm DNA damage. Oxidative DNA damage in sperm measured using 8OHdG was correlated with cadmium in seminal plasma in 56 non-smoking study participants in China (Xu *et al*., 2003). Increased DNA fragmentation in sperm measured using SCSA was correlated with blood lead levels in 80 battery plant workers in Taiwan (Hsu *et al*., 2009).

Air pollution

High levels of air pollution resulting from coal combustion was associated with increased sperm DNA fragmentation measured using SCSA in a longitudinal study of 36 men from the Czech Republic (Rubes *et al*., 2005). The components of the air pollution were not identified but blood metals were not elevated in this group and subsequent studies implicated reactive intermediates of carcinogenic polyaromatic hydrocarbons present in the particulate fraction of the air pollution (Rubes *et al*., 2007).

Although studies of each of these contaminants are limited, the accumulated epidemiologic evidence to date suggests exposures in our everyday environment can adversely affect the genetic integrity of the spermatozoon. However, the array of findings raises a number of questions about the underlying mechanisms through which environmental agents affect sperm production, structure and integrity. The future of this work relies largely on the field's ability to standardize methods of exposure and outcomes and its ability to work across clinical, epidemiologic and basic science disciplines to ask the right questions.

Among the environmental exposure studies to date, methods vary widely with respect to how exposures are defined and how sperm DNA damage is measured. Sound replication of research findings will require both valid and reliable exposure assessment measures, including evidence that the contaminant actually reaches the testis or comes into contact with sperm and validated methods for determining sperm DNA damage. In this regard, methods for detecting sperm DNA adducts that can be attributed to sources would be particularly informative. Epidemiologists are relying on clinical and basic scientists to establish the validity and precision of sperm DNA damage assays and to clarify what is actually being measured (for example, stability versus chromatin integrity). Once an assay makes its way into the reproductive biology literature, environmental epidemiologists may be quick to adopt its use as a meaningful outcome for reproductive health studies. Similarly, epidemiologic studies are designed to test exposure and outcome associations; however, there is reliance on basic science and toxicologic studies for insight into biologically plausible mechanisms that drive exposure-response relationships. In a reciprocal manner, basic scientists look to the epidemiologic data to determine whether associations they see in the lab are observed among humans in the general population or in clinical settings. Epidemiological studies seek to establish associations in large, unbiased and statistically meaningful samples of people. Together, well designed epidemiology and toxicology studies can inform clinicians about risks to sperm integrity posed by environmental contaminants and help them to advise their patients about avoiding such exposures.

Sperm DNA repair in oocytes, epigenetics and the potential consequences of faulty repair

Between the moment the spermatozoon fuses with the secondary oocyte and the first cleavage division (a period of 16–20 h in the mouse and 18–27 h in humans), the highly condensed sperm nucleus unfolds, exchanging protamines for maternal histones and assumes the interphase chromatin configuration in the male pronucleus that is necessary for the semiconservative DNA replication in preparation for the first mitotic division of embryonic development. The period of pronuclear formation in the zygote is unique in a number of features.

Sperm derived nucleosomal chromatin contributes to paternal zygotic chromatin. Using a heterologous ICSI system with human sperm and mouse oocytes, paternal nucleosomes marked by the replication dependent histone isoforms H3.1/3.2 were present in the G1 stage male pronuclei (Van Der Heijden *et al*., 2008). Additionally, examination of human multi-pronuclear zygotes showed these replication variants in paternal chromatin prior to DNA replication. This suggests that the epigenetic program originating from the paternal chromatin has the potential to be transmitted into further embryonic development (see section: Sperm epigenetics).

At least in the mouse, many post-translational histone modifications are asymmetrically present between the male and female pronucleus. Particularly in early G1 phase there are clear differences among histone N tail Lysine methylations (Van Der Heijden *et al*., 2005). Although not as extensively researched, the same pattern is visible in humans (Van Der Heijden *et al*., 2009). This phenomenon is known as epigenetic asymmetry and demonstrates the very different nature of chromatin between the sexes, as a consequence of gamete specialization, although this does not necessarily hold at the level of each gene.

Double strand DNA breaks that are induced during the nucleosome to protamine exchange in elongating spermatids may lead to residual breaks in functioning spermatozoa, although this remains to be determined. Also, and especially in the epididymis, DNA damage could originate from external factors, notably ROS. In the mouse, it has been observed that repair of DNA breaks induced by irradiation is incomplete at the first cleavage division, as the level of chromosome aberrations could be influenced by repair inhibitors at the zygote stage (Matsuda *et al*., 1989). Hence, integrity of the paternal genome also depends on the capacity of the oocyte to recognize DNA damage and repair it.

DNA repair before zygotic S-phase

Humans exhibit a relatively high load of reciprocal translocations (Bonduelle *et al*., 2002), which are mainly of paternal origin. Because a DNA double strand break is a prerequisite for a reciprocal translocation to arise, one deduces that these must be repaired during the zygotic cell cycle. The decondensation of paternal chromatin after gamete fusion is followed by a phase of recondensation, which in the mouse can be monitored by detection of Histone3 Serine10ph (H3S10ph). In the recondensation stage, 1 h after sperm fusion,

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

double strand breaks can be visualized by chromatin domains positive for phosphorylation of Serine 139 of H2AX (called gamma H2AX). Spontaneously, gammaH2AX foci are visible at this stage in low numbers and only in the paternal chromatin. However, using one genetic source of sperm and four maternal oocyte genotypes, the frequency of DNA single strand breaks was strongly dependent on the maternal genotype. Mice with either the BALB/c hypomorphic DNA.PKcs allele or the very low activity scid DNA.PKcs allele, had the highest averages of around three breaks per male complement, which was about three times the frequency found in B6/CBA hybrid oocytes (Derijck *et al*., 2008). The kinase DNA.PKcs is the key enzyme of the Non-Homologous End Joining (NHEJ) double strand DNA repair pathway. Subsequently, sperm were irradiated with a standard dose of 3 Gy a dose that damages sperm DNA. Sperm DNA damage translated into additional gamma H2AX foci that in number were highly correlated with the frequencies reported above, and so reflect DNA.PKcs activity. This experiment demonstrated that in the absence of normal DNA.PKcs alleles in oocytes, the capacity to repair DNA double strand breaks, either from a damaged sperm or from a post fusion paternal chromatin remodelling mechanical defect, is affected (Derijck *et al*., 2008).

Findings in mice have been partly confirmed in human-mouse heterologous ICSI experiments. When sperm samples from oligo-astheno-teratozoospermic men were compared with normozoospermic donors, the fraction of nuclei without damage was lower (Derijck *et al*., 2007). Again, these findings relate to a fraction of breaks showing slower repair that can be both of prefertilization origin or produced post-fertilization by an interaction between the male chromatin and the female chromatin remodelling environment on the road to pronucleus formation. The observation that motile ejaculated sperm with normal morphology can generate abnormal gammaH2AX signalling patterns up to complete fragmentation supports the contention made earlier that sperm with normal appearance may nevertheless carry DNA damage.

Cytogenetic analysis of the male and female chromosome complements at the first cell cycle has been used to demonstrate the mutagenic effect of sperm irradiation. Abnormalities in first metaphase spreads revealed primarily chromosome type aberrations (inversions, translocations, fragments, etc.). These abnormalities result when the DNA repair takes place before S-phase such that the rearranged chromosomes and chromosomal fragments are faithfully copied. In such studies scid oocytes (NHEJ defect oocytes) exhibited about twice the number of chromosome abnormalities than controls (Derijck *et al*., 2008), again implying that DNA double strand break repair is executed before S-phase.

DNA repair in the zygotic S-phase

During S-phase, when the replication fork encounters a single strand break, or more likely a base modification that requires excision repair, a double strand break is created in the replication fork. These sites can also be visualized by gammaH2AX as well as by a number of repair proteins among which is RAD51, a key player of the homologous recombination repair (HRR) pathway. A possible consequence of a stalled replication fork is a non-homologous chromatid exchange (a quadriradial) that at zygotic mitosis segregates into a normal chromosome complement and a reciprocal translocation. RAD51 has been visualized in the pronuclei from the beginning of the S-phase until chromosome contraction at mitotic metaphase (Derijck *et al*., 2008).

In mice with zygotes derived from genetically handicapped oocytes, especially in the HRR pathway (by the RAD54, RAD54B knock-out mutations) but also in the NHEJ pathway, the zygotes were extremely sensitive to the mutagen 4NQO (4-Nitroquinoline 1-oxide, a UV mimic agent). Low doses of 4NQO resulted in chromatid exchanges especially in the male pronucleus whereas higher doses caused a blockage at the pronucleus stage. These and other experiments using irradiation of zygotes before entering the S-phase showed that for all genotypes but especially for HRR mutants, the male pronuclei contain higher amount of RAD51 foci than female ones. This would indicate that the combination of damaged sperm and oocytes with suboptimal DNA repair (a possible scenario for suboptimal oocytes after ovulation induction) favours reciprocal translocation induction in the paternal chromosome complement.

Summarising, it is clear that we are only just obtaining an insight into the complexities of the DNA repair mechanisms in the early zygote. The data overwhelmingly suggest that an extra repair effort from the oocyte is required for damaged male chromatin. In compromised oocytes this may not be available.

Assay uncertainties: refinements on-going

How do we assess sperm DNA damage?

The methods discussed here are adaptations of techniques originally developed and validated for investigation of DNA in somatic cells. A crucial question is whether these adaptations are sufficient to enable reagents to get access to the compacted sperm DNA without inducing damage? Furthermore, it is essential that a method aimed at revealing sperm DNA disorders can compensate for the varying changes in the sperm chromatin structure occurring *in vitro* after ejaculation (Kvist *et al*., 1988).

The methods designed to detect sperm DNA disorders primarily include (i) Specific detection of free DNA ends ('nicks') by enzymatic incorporation of marked nucleotides (TUNEL). (ii) Detection of DNA fragments by gel embedded single-cell electrophoresis after extended lysis of all nuclear proteins binding DNA (Comet) under neutral (detects double strand) and alkaline (double strand and single strand breaks) conditions. (iii) Detection of green fluorescence as a measure of intact double stranded DNA [Acridine Orange (AO) tests] combined with the detection of red fluorescence as a measure of acid induced denaturation which occurs preferentially at sites of pre existing DNA strand breaks.

An important issue with all these methods is whether the treatments used to prepare the sperm may themselves induce DNA damage. For example, in the comet assay it is typically necessary to include thiols, which are strongly reducing, to elute protamines attaching to and hindering fragmented DNA from migrating as a comet. Does this protamine S–S reduction induce DNA damage, possibly secondary to release of physical constraints on the DNA?

With respect to the AO test using flow cytometry, the red emitted fluorescence may not be limited to pre-existing DNA damage. The assay uses acid treatment to denature damaged DNA which then reacts with AO and fluoresces red. It is possible that sperm with

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

inherently lower structural stability may be more accessible to the acid and more prone to acid induced DNA denaturation, even in the absence of pre-existing strand breakage. An example is that caput spermatozoa with condensed but unstabilized chromatin were far more sensitive to acid induced DNA-denaturation than mature cauda stallion spermatozoa (Dias *et al*., 2006). Thus, there is a need to further understand how the variability and changes in the degree and type of chromatin stabilization influence the outcome of the AO test to control for potential false negative and false positive results. This need is illustrated by the observations that (i) a high DNA fragmentation index (DFI %) among spermatozoa in raw semen was related to low success after intrauterine insemination (IUI) (Bungum *et al*., 2007), but that (ii) the prepared sperm populations that were actually used for the insemination all had low (4–6%) and normal DFI% (Bungum *et al*., 2008). Thus the 'negative impact' of originating from an ejaculate with >30% DFI is associated with the selected sperm population but hidden to the investigator as a 'falsely' normal value for DFI. For the diagnostic value for the individual patient further methodological work is needed to distinguish whether (i) a high DFI means increased vulnerability to acid and other exposures or true DNA damage that can be transmitted to future generations and (ii) a low DFI means a false negative result or no DNA damage.

To adapt the comet assay for spermatozoa, the compact status of the sperm chromatin must again be broken down using procedures that may also induce damage to the DNA. There are no standardized protocols for these procedures, but two methods have emerged: alkaline and neutral conditions. There are few studies relating this assay to clinical fertility status. Therefore, clinically robust cut-off levels remain to be established. As with the TUNEL assay, the accessibility of the DNA can be a limiting factor which needs to be addressed.

Interpretation of any test may be confounded by the presence of dead cells when these tests are performed on unprocessed semen. Dead cells contain fragmented DNA and may bias the overall results. As with all assays examining the quality of semen, a key consideration is the number of cells examined. Counting higher numbers of cells allows greater accuracy (WHO 1999) and as such any assay based on counting a low number of cells will have significantly wide confidence limits. An advantage of flow cytometry, which can be used for TUNEL and SCSA, is that large number of cells can be assessed thereby providing greater accuracy (as long as an account is made for the influence of non-sperm and dead cells).

Other tests revealing DNA accessibility

Chromomycin A3 is a compound that can bind to DNA and fluoresce. Binding to DNA is competitive with protamines and CMA3 fluorescence has therefore been interpreted as evidence for poor protamination (Bianchi *et al*., 1993). CMA3 fluorescence is certainly evidence of CMA3 binding to DNA, but this could also be due to increased access to sperm DNA due to assay procedures that may also cause a loosening between DNA and protamines (relative deprotamination). An alternative interpretation would therefore be that sperm with a high degree of CMA3 fluorescence possess DNA that is more easily accessed (and thus, more susceptible to injury), as a result of the relative deprotamination of the chromatin.

The aniline blue test is based on the detection of lysine residues with aniline blue as a measure of an excess of histones remaining bound to the sperm DNA (Dadoune *et al*., 1988).

Should DNA tests be based on the whole semen population or the subpopulation prepared for clinical use?

Whether DNA damage tests should be performed before or after sperm preparation depends on the purpose of the investigation. If the aim is to predict the potential for ART success, there is some controversy depending on the tests used. If the SCSA assay is employed, studies suggest that whole semen is more predictive (Larson *et al*., 2000; Bungum *et al*., 2007, 2008) whereas the TUNEL assay has been shown to be discriminative for clinical pregnancy using either raw semen or cohorts of spermatozoa prepared by density centrifugation for clinical use (Duran *et al*., 2002; Borini *et al*., 2006).

Whether we measure DNA from sperm taken from whole semen or isolated subpopulations, we cannot expect one single parameter to provide an absolute criterion for fertility or infertility. As for assessment of the diagnostic and prognostic value of traditional semen analysis, our expectations of sperm DNA testing are excessive. A successful ART outcome will also depend on many other traits of sperm quality e.g. capacity to fuse with the oolemma as well as the influences of the oocytes, uterine receptivity and maternal immune system competence.

The usefulness of animal studies

Animal studies are very important to our understanding of the basic biological mechanisms and the consequences of disruption of key processes. These can range from basic fertility studies, where experiments for example in cattle allow multiple inseminations and thus provide valuable preliminary information on potential diagnostic and prognostic assays (Ballachey *et al*., 1987; Amann and Hammerstedt, 2003), to developing critical models that offer the ability to perform multi-generational analyses of the consequences of ART. For ART, interpretation of animal studies depends upon an understanding of the differences in animal and human ICSI/IVF. We highlight only a few examples to illustrate how important such studies can be for predicting the future consequences of human ART. As the first example, injecting oocytes with spermatozoa with demonstrated DNA lesions as measured by SCSA has caused multi-generational effects (Fernandez-Gonzalez *et al*., 2008). This highlights possible long-term consequences of using sperm with compromised DNA integrity. Two further studies indicate the usefulness of animal models for identifying important areas of research in human ART. In the first, infertile Hook/Hook mice were used to obtain sperm for ICSI (Ward, 2005). These mice produce severely deformed spermatozoa, yet when these sperm were used for ICSI, apparently normal mice were born. More importantly, the severity of the morphological deformations did not increase in three successive generations. A second study however suggested a potential unexpected problem with ART (Collier *et al*., 2009). The clearance of steroids by the placenta was very different in offspring generated by ICSI compared with those generated by normal mating. This points to a potentially important problem that could be monitored in human pregnancies.

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

The relative ease with which animal studies in ART can be performed compared with the same investigations in human patients suggests an obvious need for the expansion of this particular type of research. The dilemma that the field faces is that it is ethically not possible to perform many of the necessary experiments to ensure that ART is completely safe, yet we have an obligation to do some assessment. Animal models can provide a step in this direction.

Clinical uncertainties: the urgency for a robust clinical test

The study of sperm DNA damage is highly relevant in the era of ART, particularly ICSI, because (i) these technologies bypass the barriers of natural selection, (ii) subfertile men possess substantially more sperm DNA damage than do fertile men and (iii) experimentally, sperm DNA damage has been shown to impact negatively on ICSI embryo development, pregnancy rates and offspring health (Ahmadi and Ng, 1999, Fernandez-Gonzalez *et al*., 2008; Zini *et al*., 2008).

There is good evidence to show that infertile men possess substantially more sperm DNA damage than do fertile men although a small percentage of spermatozoa from fertile men also possess detectable levels of DNA damage (Kodama *et al*., 1997; Evenson *et al*., 1999, Spanò *et al*., 2000; Zini *et al*., 2001). The etiology of sperm DNA damage is multi-factorial and may be due to primary testicular or secondary (e.g. environmental) factors. Ultimately, sperm DNA damage is believed to be the result of aberrant protamine expression, excessive ROS generation and abortive apoptosis during spermatogenesis (de Yebra *et al*., 1993; Carrell and Liu, 2001; Sakkas *et al*., 2003, Aitken *et al*., 2009; see also sections: Mechanisms of DNA damage in male germ cells and spermatozoa and The nature of the DNA damage exhibited by human spermatozoa).

Primary testicular factors that may disrupt spermatogenesis and spermiogenesis and subsequently lead to sperm DNA damage include ageing, congenital abnormities (cryptorchidism), genetic defects and idiopathic abnormalities. Extrinsic factors that may cause sperm DNA damage include drugs (e.g. chemotherapy), cigarette smoking, genital tract inflammation, testicular hyperthermia and varicoceles.

Epidemiologic studies of healthy men have demonstrated that the level of sperm DNA damage increases with advancing age (Spano *et al*., 1998; Wyrobek *et al*., 2006). Studies of infertile men have also generally shown that sperm DNA damage increases with advancing age (Moskovtsev *et al*., 2006; Vagnini *et al*. 2007; Winkle *et al*., 2009). These findings are in keeping with the age-dependent decline in semen parameters (Sartorius and Nieschlag, 2009).

Men with cancer (e.g. Hodgkin's lymphoma and testicular cancer) typically have poor semen quality and sperm DNA damage even prior to cancer-specific therapy (O'Flaherty *et al*., 2008). They may then experience further testicular damage with the cancer therapy (chemotherapy, radiation; Fossa *et al*., 1997; Morris, 2002). The recovery of spermatogenesis may occur months to years after therapy, but evidence of sperm DNA damage may often persist beyond that period (Fossa *et al*., 1997). Patients who are scheduled to undergo definitive cancer therapy (surgery, chemotherapy and/or radiation) are strongly encouraged to cryopreserve sperm for future use (Lee *et al*., 2006).

Cigarette smoking is associated with lower sperm counts and motility, and an increase in abnormal sperm forms and sperm DNA damage (Spano *et al*., 1998; Potts *et al*., 1999). It is postulated that smoking increases leukocyte-derived ROS production with subsequent adverse effects on mature sperm (Potts *et al*., 1999). Smokers should be counselled to stop smoking.

Post-testicular genital tract infection and inflammation (e.g. epididymo-orchitis, prostatitis) can cause leukocytospermia, and have been associated with increased levels of semen ROS and subsequent sperm DNA damage (Spano *et al*., 1998; Erenpreiss *et al*., 2002). Treatment of these genital tract infections with antibiotics may improve sperm DNA damage (Moskovtsev *et al*., 2009).

Testicular hyperthermia has been shown to cause sperm DNA damage and an increase in the histone to protamine ratio in experimental studies (Sailer *et al*., 1997, Banks *et al*., 2005). Clinically, there is limited evidence to demonstrate a relationship between hyperthermia and sperm DNA damage, however, an association between hyperthermia and reduced male fertility potential has been reported (Thonneau *et al*., 1998; Evenson *et al*., 2000). Certain behaviours (e.g. hot baths, saunas) and occupations (e.g. welders, bakers, prolonged driving) are associated with increased scrotal temperatures (Jung *et al*., 2002) and these may cause sperm DNA damage. Although men should be counselled to minimize any activity that can increase scrotal temperature, there is only limited evidence to show that this will specifically reduce sperm DNA damage.

Varicoceles have been associated with sperm DNA damage and the damage has been related to levels of oxidative stress in the semen of these infertile men (Saleh *et al*., 2003). However, it is unlikely that sperm DNA damage is specific to men with varicocele as men without varicocele (e.g. idiopathic infertility) can also possess high levels of DNA damage (Zini *et al*., 2001). Three studies have shown that varicoceletomy may reduce the levels of sperm DNA fragmentation (measured by SCSA or TUNEL assay). However, these studies are all small, uncontrolled, retrospective in design and have only tested one post-operative sperm sample (Zini *et al*., 2005; Sakamoto *et al*., 2008; Werthman *et al*., 2008). As such, the beneficial effect of varicocelectomy remains to be verified with larger, prospective studies.

It has been proposed that sperm DNA damage may be caused by seminal oxidative stress resulting from antioxidant deficiency, although the evidence in this respect is largely indirect. As such, a number of investigators have evaluated the effects of dietary antioxidant supplementation on sperm DNA integrity. Most of these clinical studies have evaluated men with high levels of sperm DNA damage. In these men, treatment with antioxidant supplements is generally associated with reduced levels of sperm DNA damage and/or improved fertility potential (Kodama *et al*., 1997; Greco *et al*., 2005a, b; Menezo *et al*., 2007; Tremellen *et al*., 2007; Gil-Villa *et al*., 2008). However, these dietary antioxidant studies were small and have not evaluated the mechanism of action of antioxidants: the only end-point that was measured is the integrity of the sperm DNA or pregnancy rate. Moreover, most studies evaluated the effects of a short treatment course (with no long-term follow-up), were not randomized and failed to include a placebo-control group. As such, vitamin supplements (antioxidants) can be offered to men with the understanding that limited clinical outcome data are not yet available to support their use.

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

Several clinical studies have examined the relationships between sperm DNA damage and reproductive outcomes in the context of natural and ART pregnancies. on the basis of a systematic review and meta-analysis of these studies, sperm DNA damage was found to be associated with lower natural, IUI and IVF pregnancy rates, but not with ICSI pregnancy rates (Collins *et al*., 2008; Zini and Sigman, 2009). Interestingly, the literature strongly suggests that sperm DNA damage is associated with an increased risk of pregnancy loss in those couples undergoing IVF or ICSI (Zini *et al*., 2008). Surprisingly there are no data relating sperm DNA damage to late fetal development or post-natal health in humans (Zini *et al*., 2008). Nonetheless, the true clinical utility of sperm DNA damage assays remains to be firmly established as the available clinical studies are generally small, heterogeneous and poorly designed, and many do not control for female factors (Collins *et al*., 2008; Zini and Sigman, 2009).

While current data suggest that impaired sperm DNA integrity may have the greatest effect (and hence, greatest clinical utility) on IUI pregnancy rates and on pregnancy loss following IVF and ICSI, larger (adequately powered), properly designed and controlled prospective studies are absolutely required to confirm these results. An evaluation of the impact of sperm DNA damage on late fetal and post-natal health is also vital.

Summary and recommendations

Recommendation 1: Fundamental research is urgently required

Significant and fundamental questions remain to be answered as part of a detailed understanding of the basic structure of chromatin and its repackaging during spermatogenesis, sperm maturation, ejaculation and during unpackaging in the oocyte. Although we do know how sperm chromatin packaging differs from that of somatic cell chromatin, we do not understand the specifics of how that packaging occurs. We are at the beginning of our understanding and uncertain where in the lifecycle of the cell the DNA damage originates, and uncertain of the causes (e.g. oxidative in nature) or the nature of the damage (e.g. single and/or double strand breaks and/or DNA cross linking). Other basic questions remain unanswered: does the origin and nature of the damage suggest less/more severe consequences? How does the oocyte recognize and repair the damage? Is there a threshold of repair? Is there a degree of damage beyond the oocyte's ability to repair? Additionally, exciting areas are now emerging such as the presence of histones, mature mRNAs and newly described microRNAs in sperm chromatin. Fundamental research is absolutely required to address the key questions.

Recommendation 2: Standardization of clinical assays

There are clear differences in protocols assessing DNA integrity and surprisingly a general lack of awareness of adequate controls both in clinical and epidemiological research. Due to the lack of standardization in methods, it is difficult to determine whether variations in findings are real (related to biology) or due to differences in method. Presently, none of the protocols appear to address the problem of different types of chromatin stabilization. Standardized methods that allow comparison of results from different laboratories are urgently required. For a correct interpretation of data, it is also essential to understand and control for sperm chromatin changes occurring after ejaculation and to distinguish between genuine DNA damage and artifacts due to lack of reagent access to DNA.

Only with standardized protocols and appropriate external quality control (EQA) is it reasonable to implement findings worldwide. For clinically useful cut-off limits, it is also a requirement that the parameter can distinguish between affected and unaffected individuals. To evaluate that, correlations are not adequate—predictive values, likelihood ratios as well as odds' ratios should be calculated to validate the usefulness of a certain parameter. ROC curves can be used to identify possible cut-off levels. Properly controlled studies with sufficiently high numbers of participating patients are more likely to be multicentre studies, which of course require standardized methods and EQA.

An EQA scheme should be developed, similar to that for Basic Semen Analysis run by the ESHRE Special Interest Group in Andrology.

Recommendation 3: Animal models

Models using animal studies should be developed to make predictions for long-term clinical outcomes of ART. Animal models offer the ability to perform multi-generational analyses of the consequences of ART, and to identify potentially hazardous molecular and biological affects of ART. We have highlighted several examples to illustrate how important animal studies can be for predicting the future consequences of human ART. The relative ease with which animal studies in ART can be performed compared with the same investigations in human patients suggests an obvious need for the expansion of this particular type of research.

Recommendation 4: High quality clinical data is urgently required

While current data suggest that impaired sperm DNA integrity may have the greatest effect (and hence, greatest clinical utility) on IUI pregnancy rates and on pregnancy loss following IVF and ICSI, significantly larger (adequately powered), properly designed and controlled prospective studies are absolutely required to confirm these results. Future studies should also aim to assess the relationships between sperm DNA damage and late reproductive outcomes (pregnancy loss, delivery rate and neonatal health) in view of the worrisome post-natal effects observed in animal studies. Sound clinical strategies for sperm DNA testing and subsequent counselling of couples seeking infertility therapies can only be developed after such studies have been undertaken. It is likely that to perform these trials large multicentre studies will be required.

Recommendation 5: Long-term follow-up of art children

It is essential that long-term comprehensive follow-up studies on ART children are performed to ascertain the safety of the procedures we are currently using. To date, there is a paucity of such studies. In general those available have limited power and do not provide comprehensive outcomes. We recommend that follow-up studies of children are performed as a matter of urgency and should include, where

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

appropriate, cross linking of databases. It is likely that with increasing developments in ART such follow-up studies will need to be continually updated and will involve international consortia.

The requirement for dedicated funding for research in reproductive medicine and infertility

We have identified real progress and presented further fascinating challenges. However, a fundamental impediment to advancement over the past three decades has been the absence of reproductive medicine and infertility research as a strategic priority for national governments and agencies. This has resulted in a paucity of funding. A prerequisite of achieving the above recommendations is for national and international agencies to realize the importance of both basic and clinical research in this area and, to deliver substantial long-term financial support. We anticipate that identifying areas for future research is an essential starting point in meeting this challenge.

Authors' Roles

Following extensive and detailed discussions at the consensus workshop, key areas to be addressed were identified (biological, assay and clinical uncertainties). Each author wrote a section related to their expertise addressing the above area(s). All contributed equally to the final drafting, editing and presentation of the paper and take equal responsibility. C.L.R.B. coordinated the construction of the manuscript and is the first author. All other authors are listed alphabetically.

Funding

Financial support from the Swedish Research Council and ESHRE.

References

Ahmadi A, Ng SC. Fertilizing ability of DNA-damaged spermatozoa. *J Exp Zool* 1999;**284**:696–704.

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

Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. In: Cheng CY (ed). *Molecular Mechanisms in Spermatogenesis*. Landes Bioscience, 2007.

Aitken RJ, De Iuliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. *Int J Androl* 2009;**32**:46–56.

Amann RP, Hammerstedt RH. Detection of differences in fertility. *J Androl* 2002;**23**:317–325.

Aoki VW, Liu L, Jones KP, Hatasaka HH, Gibson M, Peterson CM, Carrell DT. Sperm protamine 1/protamine 2 ratios are related to *in vitro* fertilization pregnancy rates and predictive of fertilization ability. *Fertil Steril* 2006a;**86**:1408–1415.

Aoki VW, Emery BR, Liu L, Carrell DT. Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. *J Androl* 2006b;**27**:890–898.

Auroux M, Dulioust E, Selva J, Rince P. Cyclophosphamide in the F0 male rat: physical and behavioral changes in three successive adult generations. *Mutat Res* 1990;**229**:189–200.

Badouard C, Ménézo Y, Panteix G, Ravanat JL, Douki T, Cadet J, Favier A. Determination of new types of DNA lesions in human sperm. *Zygote* 2008;**16**:9–13.

Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. *Biol Reprod* 1987;**36**:915–925.

Banks S, King SA, Irvine DS, Saunders PT. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. *Reproduction* 2005;**129**:505–514.

Bennetts LE, De Iuliis GN, Nixon B, Kime M, Zelski K, McVicar CM, Lewis SE, Aitken RJ. Impact of estrogenic compounds on DNA integrity in human spermatozoa: evidence for cross-linking and redox cycling activities. *Mutat Res* 2008;**641**:1–11.

Bian Q, Xu LC, Wang SL, Xia YK, Tan LF, Chen JF, Song L, Chang HC, Wang XR. Study on the relation between occupational fenvalerate exposure and spermatozoa DNA damage of pesticide factory workers. *Occup Environ Med* 2004;**61**:999–1005.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biol Reprod* 1993;**49**:1083–1088.

Biermann K, Steger K. Epigenetics in male germ cells. *J Androl* 2007;**28**:466–480.

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. Sequence of ejaculation affects the spermatozoon as a carrier and its message. *Reprod Biomed Online* 2003;**7**:440–448.

Björndahl L, Kjellberg S, Kvist U. Ejaculatory sequence in men with low sperm chromatin-zinc. *Int J Androl*. 1991;**14**:174–178.

Boissonneault G. Chromatin remodeling during spermiogenesis: a possible role for the transition proteins in DNA strand break repair. *FEBS Lett* 2002;**514**:111–114.

Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A, Liebaers I. Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. *Hum Reprod* 2002;**17**:2600–2614.

Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, Coticchio G. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod* 2006;**21**:2876–2881.

Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, Giwercman A. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod* 2007;**22**:174–179.

Bungum M, Spanò M, Humaidan P, Eleuteri P, Rescia M, Giwercman A. Sperm chromatin structure assay parameters measured after density gradient centrifugation are not predictive for the outcome of ART. *Hum Reprod* 2008;**23**:4–10.

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

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. The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. *Int J Androl* 2008;**31**:537–545.

Chabory E, Damon C, Lenoir A, Kauselmann G, Kern H, Zevnik B, Garrel C, Saez F, Cadet R, Henry-Berger J *et al.* Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J Clin Invest* 2009;**119**:2074–2085.

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

Collier AC, Miyagi SJ, Yamauchi Y, Ward MA. Assisted reproduction technologies impair placental steroid metabolism. *J Steroid Biochem Mol Biol* 2009;**116**:21–28.

Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with *in vitro* fertilization? *Fertil Steril* 2008;**89**:823–831.

Conwell CC, Vilfan ID, Hud NV. Controlling the size of nanoscale toroidal DNA condensates with static curvature and ionic strength. *Proc Natl Acad Sci USA* 2003;**100**:9296–9301.

Dadoune JP, Mayaux MJ, Guihard-Moscato ML. Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. *Andrologia* 1988;**20**: 211–217.

De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B, Aitken RJ. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* 2009;**81**:517–524.

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

de Yebra L, Ballesca JL, Vanrell JA, Bassas L, Oliva R. Complete selective absence of protamine P2 in humans. *J Biol Chem* 1993; **268**:10553–10557.

Derijck AA, Van Der Heijden GW, Ramos L, Giele M, Kremer JA, de Boer P. Motile human normozoospermic and oligozoospermic semen samples show a difference in double-strand DNA break incidence. *Hum Reprod* 2007;**22**:2368–2376.

Derijck A, van der HG, Giele M, Philippens M, de Boer P. DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. *Hum Mol Genet* 2008;**17**:1922–1937.

Dias GM, Retamal CA, Tobella L, Arnholdt ACV, Lopez ML. Nuclear status of immature and mature stallion spermatozoa. *Theriogenology* 2006;**66**:354–365.

Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002;**17**:3122–3128.

Emery BR, Carrell DT. The effect of epigenetic sperm abnormalities on early embryogenesis. *Asian J Androl* 2006;**8**:131–142.

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.

Erenpreiss J, Hlevicka S, Zalkalns J, Erenpreisa J. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. *J Androl* 2002;**23**:717–723.

ESHRE Consensus workshop on advanced diagnostic andrology techniques. ESHRE (European Society of Human Reproduction and Embryology) andrology special interest group. *Hum Reprod* 1996; **11**:1463–1479.

ESHRE Guidelines on the application of CASA technology in the analysis of spermatozoa. ESHRE andrology special interest group. European society for human reproduction and embryology. *Hum Reprod* 1998; **13**:142–145.

European Parliament. European Parliament resolution of 21 February 2008 on the demographic future of Europe (2007/2156 (INI)). www.europarl.europa.eu. (2008, last date accessed).

Evenson DP, Darzynkiewicz Z. Melamed MR Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 1980; **210**:1131–1133.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* 1999;**14**:1039–1049.

Evenson DP, Jost LK, Corzett M, Balhorn R. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. *J Androl* 2000;**21**:739–746.

Evenson DP, Wixon R. Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA). *Toxicol Appl Pharmacol* 2005;**207**:532–537.

Fernandez-Gonzalez R, Moreira P N, Perez-Crespo M, Sanchez-Martin M, Ramirez M A, Pericuesta E, Bilbao A, Bermejo-Alvarez P, De Dios Hourcade J, De Fonseca FR *et al.* Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biol Reprod* 2008;**78**:761–772.

Filipponi D, Feil R. Perturbation of genomic imprinting in oligozoospermia. *Epigenetics* 2009;**4**:27–30.

Fossa SD, De Angelis P, Kraggerud SM, Evenson D, Theodorsen L, Clausen OP. Prediction of post-treatment spermatogenesis in patients with testicular cancer by flow cytometric sperm chromatin structure assay. *Cytometry* 1997;**30**:192–196.

Gardiner-Garden M, Ballesteros M, Gordon M, Tam PP. Histone- and protamine-DNA association: conservation of different patterns within the beta-globin domain in human sperm. *Mol Cell Biol* 1998; **18**:3350–3356.

Gil-Villa AM, Cardona-Maya W, Agarwal A, Sharma R, Cadavid A. Role of male factor in early recurrent embryo loss: do antioxidants have any effect? *Fertil Steril* 2008;**92**:565–571.

Greco E, Romano S, Iacobelli M, Ferrero S, Baroni E, Minasi MG, Ubaldi F, Rienzi L, Tesarik J. ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. *Hum Reprod* 2005a;**20**:2590–2594.

Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* 2005b;**26**:349–353.

Hales BF, Crosman K, Robaire B. Increased postimplantation loss and malformations among the F2 progeny of male rats chronically treated with cyclophosphamide. *Teratology* 1992;**45**:671–678.

Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature* 2009;**460**:473–478.

Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertil Steril* 2009; Epub ahead of print October 30.

Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S, Calafat AM. DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Hum Reprod* 2007;**22**:688–695.

Hsu PC, Chang HY, Guo YL, Liu YC, Shih TS. Effect of smoking on blood lead levels in workers and role of reactive oxygen species in lead-induced sperm chromatin DNA damage. *Fertil Steril* 2009; **91**:1096–1103.

Hud NV, Allen MJ, Downing KH, Lee J, Balhorn R. Identification of the elemental packing unit of DNA in mammalian sperm cells by atomic force microscopy. *Biochem Biophys Res Commun* 1993;**193**: 1347–1354.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA. Aitken RJ DNA integrity in human spermatozoa: relationships with semen quality. *J Androl* 2000;**21**:33–44.

Jung A, Schill WB, Schuppe HC. Genital heat stress in men of barren couples: a prospective evaluation by means of a questionnaire. *Andrologia* 2002;**34**:349–355.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997;**68**:519–524.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 2008;**93**:3199–3207.

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

Kurosaka K, Takahashi M, Watanabe N, Kobayashi Y. Silent cleanup of very early apoptotic cells by macrophages. *J Immunol* 2003; **171**:4672–4679.

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

Kvist U, Björndahl L, Kjellberg S. Sperm nuclear zinc, chromatin stability, and male fertility. *Scanning Microsc* 1987;**1**:1241–1247.

Kvist U, Kjellberg S, Björndahl L, Hammar M, Roomans GM. Zinc in sperm chromatin and chromatin stability in fertile men and men in barren unions. *Scand J Urol Nephrol* 1988;**22**:1–6.

Kwan KY, Moens PB, Wang JC. Infertility and aneuploidy in mice lacking a type IA DNA topoisomerase III beta. *Proc Natl Acad Sci USA* 2003; **100**:2526–2531.

Lewis SE. Is sperm evaluation useful in predicting human fertility? *Reproduction* 2007;**134**:31–40.

Larson KL, DeJonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod* 2000;**15**:1717–1722.

Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril* 2003;**80**:895–902.

Lee SJ, Schover LR, Partridge AH, Patrizio P, Wallace WH, Hagerty K, Beck LN, Brennan LV, Oktay K. American Society of Clinical Oncology Recommendations on Fertility Preservation in Cancer Patients. *J Clin Oncol* 2006;**24**:2917–2931.

Lefièvre L, Bedu-Addo K, Conner SJ, Machado-Oliveira GS, Chen Y, Kirkman-Brown JC, Afnan MA, Publicover SJ, Ford WC, Barratt CL. Counting sperm does not add up any more: time for a new equation? *Reproduction* 2007;**133**:675–684.

Li ZP, Li KA, Tong SY. Determination for micro amounts of nucleic acids by a resonance light scattering technique with dequalinium chloride. *Talanta* 2001;**55**:669–675.

Li Y, Lalancette C, Miller D, Krawetz SA. Characterization of nucleohistone and nucleoprotamine components in the mature human sperm nucleus. *Asian J Androl* 2008;**10**:535–541.

Long M, Stronati A, Bizzaro D, Kruger T, Manicardi GC, Hjelmborg PS, Spano M, Giwercman A, Toft G, Bonde JP *et al.* Relation between serumxenobioltic-induced receptor activities and sperm DNA damage and sperm apoptotic markers in European and Inuit populations. *Reproduction* 2007;**133**:517–530.

Marcon L, Boissonneault G. Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. *Biol Reprod* 2004;**70**:910–918.

Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, Sousa M. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. *Mol Hum Reprod* 2008;**14**:67–74.

Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. *Lancet* 2004;**363**:1700–1702.

Matsuda Y, Seki N, Utsugi-Takeuchi T, Tobari I. Changes in X-ray sensitivity of mouse eggs from fertilization to the early pronuclear stage, and their repair capacity. *Int J Radiat Biol* 1989;**55**:233–256.

Martins RP, Krawetz SA. RNA in human sperm. *Asian J Androl* 2005; **7**:115–120.

McVicar CM, McClure N, Williamson K, Dalzell LH, Lewis SE. Incidence of Fas positivity and deoxyribonucleic acid double-stranded breaks in human ejaculated sperm. *Fertil Steril* 2004;**81**:767–774.

Meeker JD, Singh NP, Ryan L, Duty SM, Barr DB, Herrick RF, Bennett DH, Hauser R. Urinary levels of insecticide metabolites and DNA damage in human sperm. *Hum Reprod* 2004;**19**:2573–2580.

Meeker JD, Barr DB, Hauser R. Human semen quality and sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides. *Hum Reprod* 2008;**23**:1932–1940.

Menezo YJ, Hazout A, Panteix G, Robert F, Rollet J, Cohen-Bacrie P, Chapuis F, Clément P, Benkhalifa M. Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. *Reprod Biomed Online* 2007;**14**:418–421.

Morris ID. Sperm DNA damage and cancer treatment. *Int J Androl* 2002; **25**:255–261.

Moskovtsev SI, Lecker I, Mullen JB, Jarvi K, Willis J, White J, Lo KC. Cause-specific treatment in patients with high sperm DNA damage resulted in significant DNA improvement. *Syst Biol Reprod Med* 2009; **55**:109–115.

Nanassy L, Carrell DT. Paternal effects on early embryogenesis. *J Exp Clin Assist Reprod.* 2008;**5**:2.

Nyboe Andersen A, Goossens V, Bhattacharya S, Ferraretti AP, Kupka MS, de Mouzon J, Nygren KG. European IVF-monitoring (EIM) Consortium, for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology and intrauterine inseminations in Europe, 2005: results generated from European registers by ESHRE: ESHRE. The European IVF Monitoring Programme (EIM), for the European Society of Human Reproduction and Embryology (ESHRE). *Hum Reprod* 2009;**24**:1267–1287.

O'Flaherty C, Vaisheva F, Hales BF, Chan P, Robaire B. Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. *Hum Reprod* 2008; **23**:1044–1052.

Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. *Lancet* 2002;**360**:772–777.

Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature* 2004;**429**:154.

Oliva R. Protamines and male infertility. *Hum Reprod Update* 2006; **12**:417–435.

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

Pittoggi C, Renzi L, Zaccagnini G, Cimini D, Degrassi F, Giordano R, Magnano AR, Lorenzini R, Lavia P, Spadafora C. A fraction of mouse sperm chromatin is organized in nucleosomal hypersensitive domains enriched in retroposon DNA. *J Cell Sci* 1999;**112**:3537–3548.

Potts RJ, Newbury CJ, Smith G, Notarianni LJ, Jefferies TM. Sperm chromatin damage associated with male smoking. *Mutat Res* 1999; **423**:103–111.

Qiu J, Hales BF, Robaire B. Damage to rat spermatozoal DNA after chronic cyclophosphamide exposure. *Biol Reprod* 1995;**53**:1465–1473.

Rignell-Hydbom A, Rylander L, Giwercman A, Jonsson BA, Lindh C, Eleuteri P, Rescia M, Leter G, Cordelli E, Spano M *et al.* Exposure to PCBs and p,p′-DDE and human sperm chromatin integrity. *Environ Health Perspect* 2005;**113**:175–179.

Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, Robbins WA, Perreault SD. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. *Hum Reprod* 2005;**20**:2776–2783.

Rubes J, Selevan SG, Sram RJ, Evenson DP, Perreault SD. GSTM1 genotype influences the susceptibility of men to sperm DNA damage associated with exposure to air pollution. *Mutat Res* 2007;**625**:20–28.

Sailer BL, Sarkar LJ, Bjordahl JA, Jost LK, Evenson DP. Effects of heat stress on mouse testicular cells and sperm chromatin structure. *J Androl* 1997; **18**:294–301.

Sakamoto Y, Ishikawa T, Kondo Y, Yamaguchi K, Fujisawa M. The assessment of oxidative stress in infertile patients with varicocele. *BJU Int* 2008;**101**:1547–1552.

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online* 2003; **7**:428–432.

Sakkas D, Seli E, Manicardi GC, Nijs M, Ombelet W, Bizzaro D. The presence of abnormal spermatozoa in the ejaculate: did apoptosis fail? *Hum Fertil (Camb)* 2004;**7**:99–103.

Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, Thomas AJ Jr. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. *Fertil Steril* 2003;**80**:1431–1436.

Sanchez-Pena LC, Reyes BE, Lopez-Carrillo L, Recio R, Moran-Martinez J, Cebrian ME, Quintanilla-Vega B. Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. *Toxicol Appl Pharmacol* 2004;**196**:108–113.

Sartorius GA, Nieschlag E. Paternal age and reproduction. *Hum Reprod Update* 2010;**16**:65–79.

Moskovtsev SI, Willis J, Mullen JB. Age-related decline in sperm deoxyribonucleic acid integrity in patients evaluated for male infertility. *Fertil Steril* 2006;**85**:496–499.

Shaman JA, Ward WS. *Sperm Chromatin Stability and Susceptibility to Damage in Relation to its Structure*. Cambridge: Cambridge University Press, 2006.

Sotolongo B, Lino E, Ward WS. Ability of Hamster Spermatozoa to Digest Their Own DNA. *Biol Reprod* 2003;**69**:2029–2035.

Spano M, Kolstad AH, Larsen SB, Cordelli E, Leter G, Giwercman A, Bonde JP. The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. *Hum Reprod* 1998; **13**:2495–2505.

Spanò M, Bonde JP, Hjøllund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish first pregnancy planner study team. *Fertil Steril* 2000;**73**:43–50.

Spano M, Toft G, Hagmar L, Eleuteri P, Rescia M, Rignell-Hydbom A, Tyrkiel W, Zvyezday V, Bonde JP. Exposure to PCB and p,p'-DDE in European and Inuit populations: impact on human sperm chromatin integrity. *Hum Reprod* 2005;**20**:3488–3499.

Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. *Biol Reprod* 1997;**56**:602–607.

Tateno H, Kimura Y, Yanagimachi R. Sonication per se is not as deleterious to sperm chromosomes as previously inferred. *Biol Reprod* 2000; **63**:341–346.

Thonneau P, Bujan L, Multigner L, Mieusset R. Occupational heat exposure and male fertility: a review. *Hum Reprod* 1998;**13**:2122–2125.

Tomsu M, Sharma V, Miller D. Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. *Hum Reprod* 2002;**17**:1856–1862.

Trasler JM. Epigenetics in spermatogenesis. *Mol Cell Endocrinol* 2009; **306**:33–36.

Tremellen K, Miari G, Froiland D, Thompson J. A randomised control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. *Aust N Z J Obstet Gynaecol* 2007; **47**:216–221.

Van Der Heijden GW, Dieker JW, Derijck AA, Muller S, Berden JH, Braat DD, van der Vlag J, de Boer P. Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote. *Mech Dev* 2005;**122**: 1008–1022.

Van Der Heijden GW, Derijck AA, Ramos L, Giele M, van der Vlag J, de Boer P. Transmission of modified nucleosomes from the mouse male germline to the zygote and subsequent remodeling of paternal chromatin. *Dev Biol* 2006;**98**:458–469.

Van Der Heijden GW, Ramos L, Baart EB, van dB I, Derijck AA, van d V, Martini E, de BP. Sperm-derived histones contribute to zygotic chromatin in humans. *BMC Dev Biol* 2008;**8**:34.

Van Der Heijden GW, van dB I, Baart EB, Derijck AA, Martini E, de BP. Parental origin of chromatin in human monopronuclear zygotes revealed by asymmetric histone methylation patterns, differs between IVF and ICSI. *Mol Reprod Dev* 2009;**76**:101–108.

Van Kooij RJ, de Boer P, De Vreeden-Elbertse JM, Ganga NA, Singh N, Te Velde ER. The neutral comet assay detects double strand DNA damage in selected and unselected human spermatozoa of normospermic donors. *Int J Androl* 2004;**27**:140–146.

Vagnini L, Baruffi RL, Mauri AL, Petersen cG, Massaro FC, Pontes A, Oliveira JB, Franco JG Jr. The effects of male age on sperm DNA damage in an infertile population. *Reprod Biomed Online* 2007; **15**:514–519.

Ward MA. Intracytoplasmic sperm injection effects in infertile azh mutant mice. *Biol Reprod* 2005;**73**:193–200.

Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S, Oehninger S. Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod* 2002;**8**:984–991.

Werthman P, Wixon R, Kasperson K, Evenson DP. Significant decrease in sperm deoxyribonucleic acid fragmentation after varicocelectomy. *Fertil Steril* 2008;**90**:1800–1804.

Windt ML, De Beer PM, Franken DR, Rhemrev J, Menkveld R, Lombard CJ, Kruger TF. Sperm decondensation and semen parameters: utilization of a simple staining technique for the evaluation of human sperm decondensation. *Andrologia* 1994;**26**:67–72.

Winkle T, Rosenbusch B, Gagsteiger F, Paiss T, Zoller N. The correlation between male age, sperm quality and sperm DNA fragmentation in 320 men attending a fertility center. *J Assist Reprod Genet* 2009; **26**:41–46.

World Health Organization. *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interactions*. 4th edn, Cambridge, UK: Cambridge University Press, 1999.

Wykes SM, Krawetz SA. The structural organization of sperm chromatin. *J Biol Chem* 2003;**278**:29471–29477.

Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW, Glaser RL, Pearson FS, Evenson D. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proc Natl Acad Sci USA* 2006;**103**:9601–9606.

Xu DX, Shen HM, Zhu QX, Xhua L, Wang QN, Chia SE, Ong CN. The assoications amon semn quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. *Mutat Res* 2003;**534**:155–163.

Zalenskaya IA, Zalensky AO. Non-random positioning of chromosomes in human sperm nuclei. *Chromosome Res* 2004;**12**:163–173.

Zalenskaya IA, Bradbury EM, Zalensky AO. Chromatin structure of telomere domain in human sperm. *Biochem Biophys Res Commun* 2000;**279**:213–218.

Ziebe S, Devroey P, State of ART 2007 Workshop Group. Assisted reproductive technologies are an integrated part of national strategies addressing demographic and reproductive challenges. *Hum Reprod Update* 2008;**14**:583–592.

Zini A, Sigman M. Are tests of sperm DNA damage clinically useful?: Pros and Cons. *J Androl* 2009;**30**:219–229.

Zini A, Bielecki R, Phang D, Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 2001;**75**:674–677.

Zini A, Blumenfeld A, Libman J, Willis J. Beneficial effect of microsurgical varicocelectomy on human sperm DNA integrity. *Hum Reprod* 2005; **20**:1018–1021.

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014

Zini A, Boman J, Belzile E, Ciampi A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: Systematic review and meta-analysis. *Hum Reprod* 2008;**23**:2663–2668.

Appendix: Workshop details

The workshop was developed by ESHRE Special Interest Group (SIG) 'Andrology' as a ESHRE Campus course entitled 'Sperm DNA: organization, protection and vulnerability—from basic science to clinical application'. The meeting took take place in the Karolinska Institute Stockholm (Sweden) and was divided into two workshops: (i) a consensus workshop on 19th–20th May 2009 for invited speakers only and (ii) an ESHRE campus course on 21st–22nd May 2009 which was an open meeting. The meeting was initiated, organized and coordinated by Lars Björndahl and Ulrik Kvist. The speakers were invited, by EHSRE, based on their expertise in the area.

Previous ESHRE workshops in Andrology have been published as ESHRE Consensus Workshop on advanced diagnostic andrology techniques. ESHRE (European Society of Human Reproduction and Embryology) andrology special interest group. *Hum Reprod* 1996;**11**:1463–1479 and ESHRE Guidelines on the application of CASA techology in the analysis of spermatozoa. ESHRE andrology special interest group. European society for human reporduction in Embryology. *Hum Reprod* 1998;**13**:142–145.

Submitted on September 24, 2009; resubmitted on November 29, 2009; accepted on December 2, 2009

Downloaded from http://humrep.oxfordjournals.org/ by guest on October 27, 2014