Molecular Human Reproduction, Vol.19, No.8 pp. 475–485, 2013
Advanced Access publication on April 1, 2013 doi:10.1093/molehr/gat025

MHR Basic science of reproductive medicine

NEW RESEARCH HORIZON ***Review***

The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies

R.J. Aitken[1,2,*], R. Bronson[3], T.B. Smith[1,4], and G.N. De Iuliis[1]

[1]Discipline of Biological Sciences and Priority Research Centre in Reproductive Science, Faculty of Science and IT, University of Newcastle, University Drive, Callaghan, NSW 2308, Australia [2]Hunter Medical Research Institute, New Lambton Heights, Newcastle, NSW 2305, Australia [3]Obstetrics and Gynecology and Pathology, Stony Brook University Medical Center, Stony Brook, NY 11794, USA

*Correspondence address. Tel: +61-2-4921-6143; Fax: +61-2-4921-6308; E-mail: john.aitken@newcastle.edu.au

Submitted on January 31, 2013; resubmitted on March 19, 2013; accepted on March 25, 2013

ABSTRACT: This article considers the origins of DNA damage in human spermatozoa, the methods that are available to monitor this aspect of semen quality and the clinical significance of such measurements. DNA damage in spermatozoa appears to be largely oxidative in nature, inversely correlated with levels of nuclear protamination and frequently associated with the activation of a truncated apoptotic pathway. DNA base adducts formed as a result of oxidative attack are released from the spermatozoa into the extracellular space through the action of a glycosylase, OGG1. This creates an abasic site, which is not resolved until fertilization because spermatozoa do not possess the molecular machinery needed to continue the base excision repair pathway. The abasic sites so generated in human spermatozoa are readily detected by SCSA or the Comet assay; however, no signal is detectable with TUNEL. This is because spermatozoa lack the enzyme (APE1) needed to create the free 3′ hydroxyl groups required by this detection system. Nevertheless, spermatozoa do eventually become TUNEL positive as they enter the perimortem. The American Society of Reproductive Medicine Practice Committee has suggested that DNA damage in spermatozoa should not be assessed because the correlation with pregnancy is inconsistent across independent studies. However, this is a straw man argument. The reason why such assays should be undertaken is not just that they reflect the underlying quality of spermatogenesis but, more importantly, that the DNA damage they reveal may have detrimental effects on the developmental normality of the embryo and the health of possible future children.

Key words: sperm / DNA damage / oxidative stress / IVF

Introduction

We have been aware that defects in the genetic constitution of the paternal germ line can influence the course of embryonic development since Weinberg's pioneering observations on the relationship between birth order and the incidence of achondroplasia in 1912 (Crow, 2000). However, it was not until the late 1980s that the potential significance of DNA damage in mature spermatozoa became apparent when Singh *et al.* (1989) demonstrated the presence of a surprisingly large number of single-strand DNA breaks in these cells. Using the alkaline Comet assay, $\sim 10^6$ to 10^7 breaks per genome were detected in human and mouse spermatozoa but not in human lymphocytes or in mouse bone marrow cells (Singh *et al.*, 1989). These authors concluded that such breaks might be physiological and related to the compaction of the entire haploid genome into just 5 μm^3, the volume occupied by a human sperm head (Lee *et al.*, 1997). Gorczyca *et al.* (1993) subsequently used the TUNEL assay to confirm that human spermatozoa possess significant numbers of DNA strand breaks and that the presence of such damage correlated with the susceptibility of the DNA to acid denaturation. In the same year Bianchi *et al.* (1993) showed that the presence of DNA damage in human spermatozoa was inversely correlated with the degree of chromatin protamination as measured using the fluorescent probe chromomycin A3 (CMA3), which is thought to compete with protamines for binding sites on the DNA. Such studies suggested that deficient sperm compaction during spermiogenesis created vulnerability in the spermatozoa to subsequent DNA damage (Manicardi *et al.*, 1995). This theme was reinforced by Evenson's group (Sailer *et al.*, 1995) in a study in which they demonstrated a correlation between the presence of spontaneous DNA nicks in the spermatozoa

[4] Present address: Program in Membrane Biology, MGH Simches Research Center, 185 Cambridge Street, Boston, MA 02114, USA

of several species, including man, and the susceptibility of the sperm chromatin to acid denaturation as measured in the sperm chromatin structure assay (SCSA).

At around this time, Fraga et al. (1991, 1996) published two important papers that gave the first clues concerning the chemical nature of the damage recorded in human spermatozoa when they observed that men characterized by low levels of antioxidant protection (because their diet was deficient in vitamin C or because they were heavy smokers) exhibited high levels of oxidative DNA damage (8-hydroxy, 2′-deoxyguanosine [8OHdG]) in their spermatozoa. Given the evidence that has subsequently emerged of the relationship between paternal smoking and cancer in the offspring (Ji et al., 1997; Lee et al., 2009), these findings have taken on a pioneering significance. They permitted, for the first time, a rational set of relationships to be proposed between environmental and lifestyle factors, oxidative DNA damage in the paternal germ line and the incidence of serious disease in the offspring. Publications on this topic have accelerated dramatically in recent years and we have now reached a point in the evolution of the DNA damage story when it is appropriate to look back at what we have learnt over the past decade in terms of the origins of the damage, methods for its robust assessment and its clinical and biological significance.

How does DNA damage in the germ line occur?

By the mid 1990s three interpretations were possible concerning the origins of DNA damage in human spermatozoa and its relationship with chromatin compaction:

(i) *it represents a causal relationship between poor chromatin compaction and DNA damage*—according to this school of thought, defects during spermiogenesis leading to poorly compacted sperm chromatin generate a state of susceptibility to subsequent DNA fragmentation at some point between spermiation and ejaculation;

(ii) *an independent relationship exists between DNA damage and poor chromatin compaction*—this model holds that protamination and DNA fragmentation are independent phenomena reflecting the underlying quality of spermiogenesis. According to this scheme, DNA nicks occur physiologically in spermatids in order to relieve the torsional stresses associated with DNA compaction but are normally labelled by gammaH2AX and resolved. However, when spermiogenesis is defective, the anticipated resolution of these nicks in late spermatogenesis does not occur and they persist in the mature gamete alongside a poorly compacted sperm nucleus (Leduc et al., 2008);

(iii) *the relationship between DNA damage and chromatin compaction is an artefact*—this interpretation is based on the fact that the assays used to detect DNA damage in these early studies depended on the use of large bulky enzymes (terminal transferases and DNA polymerases in the TUNEL and nick translation assays, respectively) to replace the target nucleotides of a DNA sequence with their labelled analogues. This strategy is perfectly effective with interphase somatic nuclei; however, the DNA in a sperm nucleus approaches the physical limits of compaction and is in a quasi-crystalline state. Thus, with this particular cell type there is a technical problem with these assays because the enzymes on which their functionality depends will inevitably experience difficulties in gaining access to the sites of DNA strand breakage. Under these circumstances, it is possible that poor compaction—due to low levels of protamination or high levels of DNA fragmentation—sufficiently relaxes the structure of sperm chromatin to permit these assays to function. Thus, DNA stand breaks may be an inherent feature of sperm chromatin, induced during the extensive nuclear remodelling associated with spermiogenesis but it is only in poorly compacted specimens that these nicks can be observed with assays dependent on the participation of enzymes, such as TUNEL. In order to elucidate which of these three interpretations is correct, we need to consider the chemical mechanisms by which DNA damage is induced in the germ line.

Cause of DNA damage: oxidative stress or endonucleases?

There are only two ways in which DNA strand breakage can occur—free radical attack and enzymatic cleavage. The finding that male infertility is associated with an increase in reactive oxygen species (ROS) generation originating from the spermatozoa and, occasionally, leukocytes (Aitken and Clarkson, 1987; Alvarez et al., 1987; Aitken et al., 1989; Baker and Aitken, 2004, 2005) suggested that an oxidative attack on the DNA backbone might be a possibility in defective human spermatozoa. Since such attacks are preferentially focused on guanine residues, the oxidative base adduct, 8-hydroxy-2′-deoxyguanosine (8OHdG), was targeted and found to be present in high amounts in the spermatozoa of infertile patients (Kodama et al., 1997). The importance of sperm preparation techniques in the genesis of this damage suggested that oxidative DNA damage could be induced in mature human spermatozoa following ejaculation; it did not have to originate in the testes or epididymis (Twigg et al., 1998b). Accordingly, oxidative DNA damage can be readily induced *in vitro*, in otherwise normal human spermatozoa, by exposure to an oxidative stress (Twigg et al., 1998a; Sierens et al., 2002; Sawyer et al., 2003), Furthermore, in careful dose-dependent studies it was demonstrated that the DNA in the sperm nucleus was more vulnerable to oxidative attack than the mechanisms regulating motility or sperm-oocyte fusion (Aitken et al., 1998). Hence, while all aspects of sperm function will ultimately succumb to oxidative stress, DNA is particularly vulnerable. As a result, it is quite feasible to imagine situations, such as paternal smoking, where the DNA is oxidatively damaged but the spermatozoa are still competent to fertilize the oocyte and deliver their damaged payload into the oocyte.

Interestingly, studies involving the chemical induction of oxidative DNA damage have emphasized that the highly compacted nuclear genome present in spermatozoa is actually quite resistant to oxidative stress when compared with somatic cells, compensating in some way for the intrinsic lack of antioxidant defence enzymes in these cells and their ineptitude at DNA repair (Sawyer et al., 2003). When the DNA is poorly compacted, this protection, which is dependent on the close association of DNA with cysteine rich protamines (Bennetts and Aitken, 2005; Enciso et al., 2011), is lost and the cells become very susceptible to oxidative DNA damage. In keeping with this model,

excellent correlations have been observed between DNA fragmentation, 8OHdG formation and impaired protamination of the sperm nucleus (De Iuliis *et al*., 2009b).

The nature of chromatin compaction in the sperm nucleus has been excellently reviewed by Ward (2010). According to this model, the DNA is compacted into doughnut-shaped toroids that contain ~50 kb of DNA in a semicrystalline state. Interspersed between these toroids are interlinker regions of DNA that form a close association with the nuclear matrix. Within the toroids, extensive creation of intra- and inter-molecular disulphide bridges within and between protamines during epididymal transit generates a chromatin structure that is relatively resistant to damage once the spermatozoa have achieved a state of maturity (Sawyer *et al*., 2003). By contrast, the inter-toroid linker regions are histone rich and are, therefore, particularly vulnerable to attack by nucleases (Villani *et al*., 2010; Ward, 2010) and, presumably, ROS. In normally compacted sperm chromatin, the DNA cleavage would, therefore, preferentially occur at the interlinker regions. However, in defective spermatozoa the chromatin is relatively histone rich (Foresta *et al*., 1992) creating additional areas of vulnerability that may extend into the toroid regions of the DNA in order to generate the close correlations that have been observed between poor protamination and DNA damage (De Iuliis *et al*., 2009b; Simon *et al*., 2011a, b). Such observations have led to a 'two-step' hypothesis of DNA damage in human spermatozoa, whereby poor protamination of sperm chromatin during spermiogenesis is held to create a state of vulnerability that is subsequently exploited in attacks on DNA integrity that are largely mediated by ROS (Aitken and De Iuliis, 2010). Such a model is clearly consistent with the correlations highlighted above between DNA fragmentation, 8OHdG formation and chromatin protamination. Indeed, these associations are so strong that oxidative damage to sperm DNA can be seen as an indirect deflection of the quality of sperm chromatin remodelling during spermiogenesis.

The idea that oxidative stress is responsible for the DNA damage observed in human spermatozoa gathered pace after 1998, with several authors confirming the presence of significantly elevated levels of 8OHdG in the spermatozoa of infertile patients and finding weak, often inconsistent, correlations ($r = \sim 0.3-0.4$) with conventional measures of semen quality (Shen *et al*., 1999; Barroso *et al*., 2000; Loft *et al*., 2003). It was also about this time that oxidative stress in defective human spermatozoa was found to be associated with markers of apoptosis including phosphatidylserine exteriorization (Barroso *et al*., 2000) and caspase activation (Wang *et al*., 2003). Although apoptosis is conventionally associated with the release of endonucleases and the widespread induction of DNA fragmentation, this is not the case with spermatozoa. In these cells, it is oxidative stress that initiates the DNA fragmentation seen in the patient population for reasons that are set out below.

Oxidative stress, apoptosis and DNA damage: a unifying hypothesis

The primary reasons why human spermatozoa suffer from oxidative stress are as numerous as they are complex and interdependent. They include a loss of antioxidant protection (Smith *et al*., 1996), the presence of free-radical-generating phagocytes in the immediate vicinity of the spermatozoa (Aitken *et al*., 1992; Saleh *et al*., 2002) and ROS generation by the spermatozoa themselves either from their mitochondria (Koppers *et al*., 2011) or, possibly, from NADPH oxidases (Aitken *et al*., 1997; Donà *et al*., 2011). In order to integrate these observations together into a single coherent scheme, it may be instructive to reflect that ROS generation by human spermatozoa can, itself, be induced by ROS in a self-perpetuating cycle (de Lamirande and Lamothe, 2009; Aitken *et al*., 2012). Thus, if spermatozoa are exposed to hydrogen peroxide or are co-incubated with free-radical-generating leukocytes, then ROS generation by human spermatozoa is stimulated (Saleh *et al*., 2002). It is also important to emphasize that oxidative DNA damage (8OHdG) is correlated with other criteria for assessing DNA damage including SCSA and TUNEL (Oger *et al*., 2003; De Iuliis *et al*., 2009a, b) and is, therefore, central to the aetiology of sperm DNA damage, not merely a facet of it. Somehow we have to integrate these findings on ROS, oxidative stress, apoptosis, chromatin compaction, oxidative DNA damage and DNA fragmentation into a single coherent scheme that accounts for most, if not all, of the observations made on this phenomenon. A possible hypothesis is advanced in Fig. 1.

The basic tenet of this hypothesis is that spermatozoa experiencing oxidative stress default to an apoptotic pathway that begins by triggering enhanced ROS generation by the mitochondria and culminates in DNA fragmentation and cell death. As indicated in Fig. 1, the pathways leading to oxidative stress are numerous. They may involve local or systemic antioxidant depletion (Aitken, 1995; Gharagozloo and Aitken, 2011), exposure to radiofrequency electromagnetic radiation or heat, (De Iuliis *et al*., 2009a), poor differentiation during spermiogenesis resulting in excess retention of residual cytoplasm (Gomez *et al*., 1996) and poor chromatin compaction (De Iuliis *et al*., 2009b), exposure to heavy metals such as cadmium (Xu *et al*., 2003), prolonged culture *in vitro* (Muratori *et al*., 2003) or reproductive toxicants of various kinds (Barratt *et al*., 2010; Aitken and Curry, 2011). The net effect of any of these sperm stressors is to initiate the intrinsic apoptotic cascade in these cells.

The trigger here is a failure to fully maintain the phosphorylation status of PI3-kinase/AKT1 (Koppers *et al*., 2011). This pathway appears to be critical to the maintenance of sperm survival because it prevents these cells from defaulting to an apoptotic state. Prosurvival factors such as insulin or prolactin serve to enhance the phosphorylation status of PI3 kinase/AKT and in this way can prolong the survival of these cells (Pujianto *et al*., 2010). However, if PI3 kinase is inhibited with compounds such as wortmannin, the cells rapidly default to an apoptotic cascade characterized by rapid motility loss, mitochondrial ROS generation, caspase activation in the cytosol, phosphatidylserine exposure on the cell surface, cytoplasmic vacuolization and oxidative DNA damage (Koppers *et al*., 2011).

The fact that this apoptotic pathway starts with the activation of mitochondrial ROS generation is significant, since it explains how a wide variety of different suboptimal conditions can culminate in a state of oxidative stress. Thus, any condition that can diminish the phosphorylation status of PI3 kinase/AKT can trigger an apoptotic response by human spermatozoa and one of the first signs that apoptosis has been induced is the release of mitochondrial ROS. From this point onwards, oxidative stress becomes a self-perpetuated

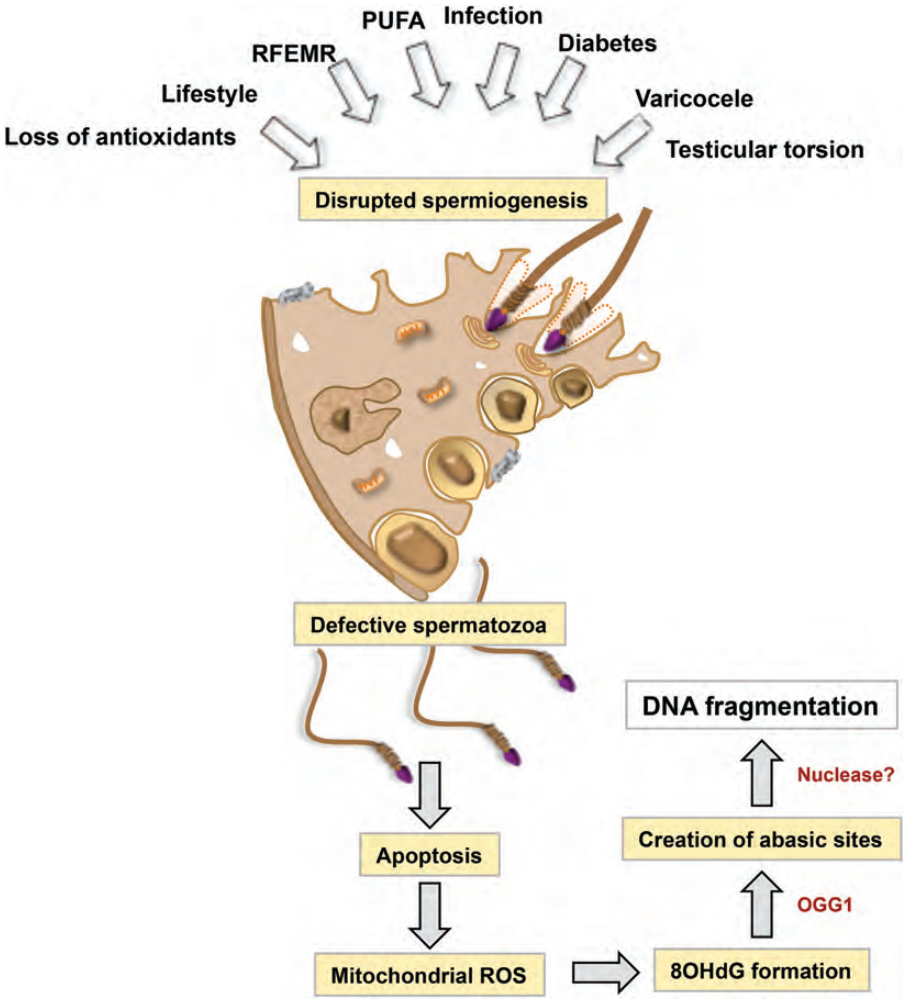

Figure 1 A general scheme for the generation of DNA strand breaks in human spermatozoa. According to this proposal a variety of genetic, metabolic, lifestyle and environmental factors can perturb the later stages of spermiogenesis resulting in the generation of defective spermatozoa characterized by poorly compacted chromatin. These defective cells will ultimately experience a state of oxidative stress driven by the mitochondrial ROS generated when they default to the intrinsic apoptotic cascade. The ROS then attack the poorly compacted chromatin generating oxidized DNA base adducts such as 8-hydroxy, 2′deoxyguanosine (8OHdG). The spermatozoon responds with oxoguanine glycosylase (OGG1) which clips the base adduct out of the chromatin generating an abasic site and a single-strand break. These changes ultimately destabilize the DNA leading to high levels of fragmentation, possibly aided by nucleases of intracellular (Sotolongo *et al.*, 2005) or extracellular (Boaz *et al.*, 2008) origin. PUFA = polyunsaturated fatty acid, RFEMR = radio frequency electromagnetic radiation.

cascade of ROS-induced ROS production from which there is no escape. As soon as oxidative stress is initiated, the high polyunsaturated fatty acid (PUFA) content of human spermatozoa ensures the rapid activation of a lipid peroxidation cascade that generates small-molecular-mass lipid aldehydes such as 4HNE, acrolein and malondialdehyde. These aldehydes are electrophilic and will rapidly form covalent bonds with the nucleophilic centres of susceptible proteins. One of the major targets for these electrophilic lipid aldehydes turns out to be the proteins of the mitochondrial electron transport chain (ETC), including succinic acid dehydrogenase (Aitken *et al.*, 2012). Adduction of these proteins interferes with the regulated transport of electrons along the ETC, leading to the adventitious formation of superoxide anion. The latter then dismutates to hydrogen peroxide, inducing the production of yet more electrophilic lipid aldehydes that again target the ETC—and so the cycle continues (Fig. 2). Hence, whether the initial insult to the spermatozoon is developmental, environmental or a consequence of some pathological process such as infection or diabetes, the net result is the activation of apoptosis and the creation of oxidative stress—all roads lead to an oxidative Damascus.

Several hours after the activation of mitochondrial ROS, other classical markers of the apoptotic cascade become expressed including caspase activation and phosphatidylserine externalization (Koppers *et al.*, 2011). The appearance of these markers should then be associated with the release of endonucleases from the mitochondria (e.g. endonuclease G) or the activation of these enzymes in the cytosol (e.g. caspase activated DNase) followed by their migration into the

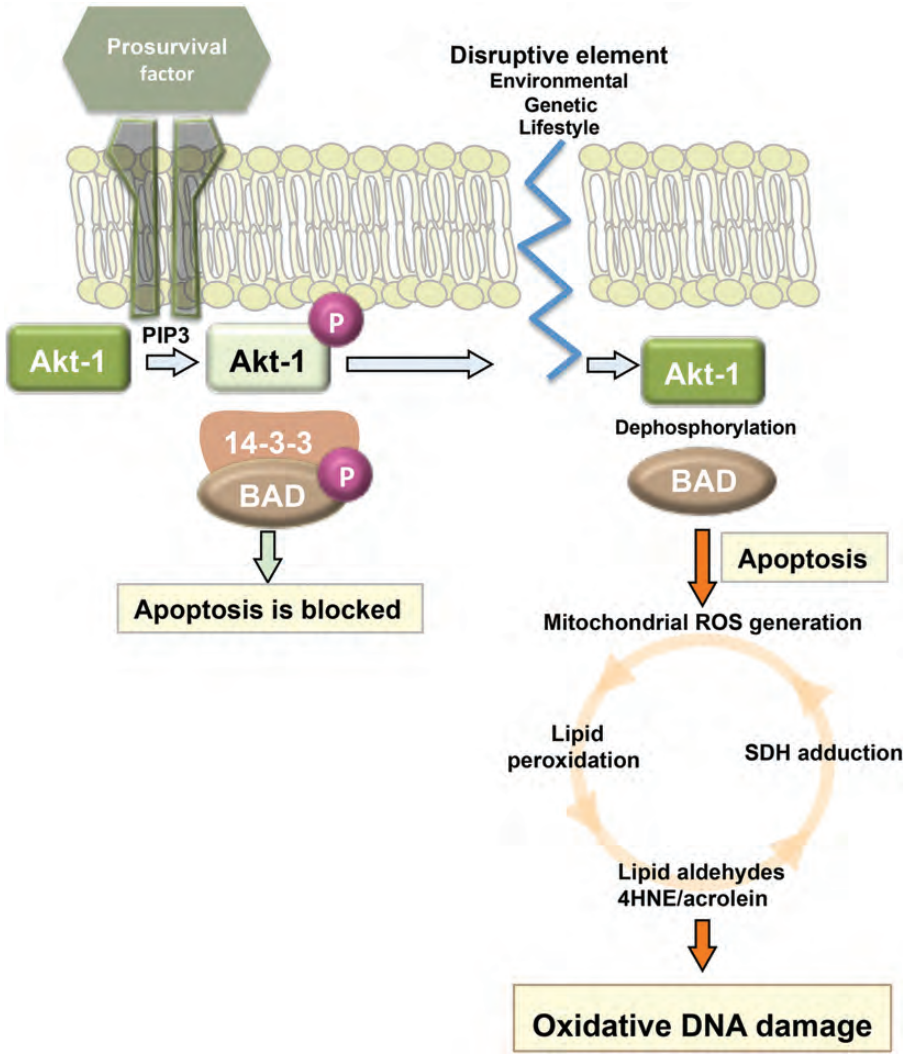

Figure 2 The intrinsic apoptotic cascade in human spermatozoa. As long as protein kinase B (AKT1) is phosphorylated, the spermatozoa are viable and potential mediators of apoptosis such as Bcl-2 associated death promoter (BAD) are held in an inactivated, phosphorylated state with their keeper protein 14-3-3. As soon as AKTI is inactivated, as a result of the absence of prosurvival factors such as insulin or the presence of disruptive elements (electromagnetic radiation, toxic metabolites, environmental pollutants), pro-apoptotic factors like BAD become activated by dephosphorylation and the intrinsic apoptotic cascade is initiated. The latter involves the generation of ROS by the mitochondria via a self-perpetuating cycle in which lipid peroxidation generates electrophilic aldehydes that bind to the mitochondrial ETC, triggering more ROS generation and further lipid peroxidation. The net result of this oxidative stress is to induce oxidative base damage in the sperm DNA. SDH = succinic acid dehydrogenase.

sperm nucleus and enzymatic cleavage of the DNA. Although this would be a rational corollary of apoptosis in a somatic cell featuring a typical interphase nucleus surrounded by a sea of cytoplasm and mitochondria, this logic does not extend to spermatozoa.

One of the latter's unique characteristics is a highly compartmentalized architecture in which the nucleus, located in the sperm head, is physically separated from the mitochondria and a majority of the cytoplasm concentrated in the sperm midpiece (Fig. 3). As a consequence of this arrangement, the endonucleases released and activated during apoptosis remain resolutely locked in the midpiece of the cell and never gain access to the nucleus (Koppers *et al.*, 2011). Even if an endonuclease did manage to gain access to the sperm nucleus, it would take some time to permeate such a dense structure and induce widespread DNA damage. The only components of the apoptotic cascade that are generated in the midpiece and can impact upon chromatin integrity in the sperm head are ROS, such as hydrogen peroxide—a powerful, membrane-permeant oxidant capable of inducing significant damage to DNA in the sperm nucleus. The small molecular mass of such oxidants also enables them to penetrate into areas of the chromatin from which nucleases such as DNAse I would be excluded because of their bulk (Villani *et al.*, 2010). These considerations explain why most of the DNA damage in human spermatozoa is oxidative (De Iuliis *et al.*, 2009b). Furthermore, if the primary lesion is a failure of normal chromatin compaction during spermiogenesis and the

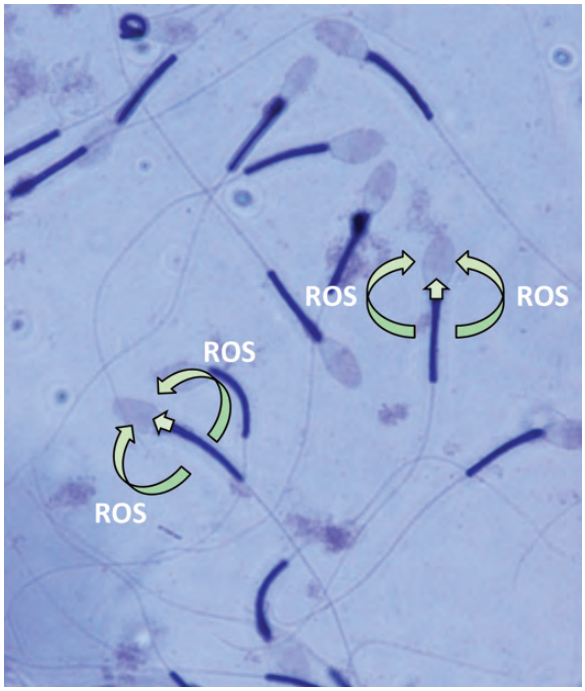

Figure 3 Spermatozoa are highly compartmentalized cells in which the nucleus is physically separated from the mitochondria and a majority of the cytoplasm. As a result, when these cells undergo apoptosis, endonucleases activated in the cytoplasm or released from the mitochondria remain resolutely locked in the midpiece of the cell (stained dark blue). The only elements of the apoptotic cascade that can impact on DNA integrity are the ROS produced by the sperm mitochondria.

oxidative attack associated with apoptosis is subsequent to spermiation, and possibly ejaculation, there may be ample opportunities to prevent or at least limit the level of oxidative DNA damage sustained by the spermatozoa through the careful use of antioxidants and appropriate sperm preparation techniques (Greco *et al.*, 2005; Aitken *et al.*, 2011; Gharagozloo and Aitken, 2011).

The only other mechanism for inducing DNA damage in human spermatozoa would be the persistence of unresolved DNA nicks from the chromatin remodelling that occurs during spermiogenesis. Under normal circumstances, the strand breaks induced by topoisomerase to relieve the torsional stresses associated with DNA compaction would become labelled with gamma H2AX and repaired (Leduc *et al.*, 2008). It is theoretically possible that if this repair process were to be impaired in any way then the strand breaks would persist in the mature gamete. Furthermore, defects in the topoisomerase system might be associated with a failure to remodel sperm chromatin adequately, leaving spermatozoa with unresolved topoisomerase-mediated strand breaks and persistent gamma H2AX foci that would also be vulnerable to oxidative stress and apoptosis. The persistence of these gamma H2AX foci would not necessarily be associated with any signs of oxidative stress, although the latter has been proposed to directly stimulate H2AX phosphorylation in human spermatozoa (Li *et al.*, 2006). If this is the case, then it will always be very difficult to determine whether the DNA damage seen in ejaculated spermatozoa is induced by oxidative stress arising during the terminal stages of spermiogenesis or following the release of the spermatozoa from the germinal epithelium at spermiation. Similarly, it will be difficult to determine conclusively whether any involvement of apoptosis in the aetiology of oxidative stress and DNA damage precedes or succeeds spermiation. Further studies of the persistence of gamma H2AX foci in human spermatozoa and their association with oxidative stress/apoptosis markers and criteria for normal spermiogenesis such as the efficiency of chromatin compaction or the retention of excess levels of sperm cytoplasm will be needed to address this point.

From a diagnostic perspective, it may not really matter when sperm DNA becomes attacked. The important question that needs to be addressed is the sensitivity and interrelatedness of the large number of assays that are currently used to detect DNA in patients' samples.

Methods for the measurement of DNA damage in human spermatozoa

In order to determine which DNA damage assay is the most appropriate for clinical screening purposes, we need to consider the biochemistry underpinning the DNA damage process and the cellular responses to this event. As discussed above, the primary attack on sperm DNA appears to be oxidative, generating high levels of the base adduct, 8OHdG, within the patient population. The spermatozoon has a limited capacity to respond to the presence of this oxidized base other than to activate the base excision repair pathway and enzymatically remove the adduct through the action of an oxoguanine glycosylase, OGG1 (Smith *et al.*, 2013). In spermatozoa, OGG1 is closely associated with DNA in both the sperm nucleus and mitochondria and is biochemically active in cleaving out 8OHdG residues, which are then released to the outside of the cell. Thus, when spermatozoa are attacked by hydrogen peroxide, 8OHdG rapidly appears in the extracellular space (Smith *et al.*, 2013). The result of OGG1 action is, therefore, to create abasic sites. The ability of alkaline conditions to create DNA strand breaks from abasic sites explains why the alkaline Comet assay is such a sensitive method for detecting oxidative DNA damage in spermatozoa (Aitken *et al.*, 1998; Irvine *et al.*, 2000; Simon *et al.*, 2011a, b). Similarly, the abasic sites created by OGG1 will destabilize the DNA making it more vulnerable to acid hydrolysis and expression of the single-stranded DNA targeted by the SCSA assay (Aravindan *et al.*, 1997).

Within the base excision repair pathway, OGG1 not only cleaves the oxidized base from the DNA but also places a nick in the phosphodiester backbone yielding a 3′ α,β-unsaturated aldehyde and a 5′deoxyribosephosphate. Apurinic endonuclease 1 (APE1) then cleaves the AP site to form a 3′-OH group adjacent to the 5′deoxyribosephosphate in preparation for the insertion of a new guanine nucleotide by polymerase β. It is the 3′-OH group created by APE1 that then serves as the target for terminal transferase employed in the TUNEL assay. A careful analysis of the base excision repair pathway in human spermatozoa has revealed that APE1 is not present in these cells (Smith *et al.*, 2013). Similarly, XRCC1, a scaffolding enzyme involved in the stabilization of the base excision repair pathway was not detected in these cells (Smith *et al.*, 2013). These

data, therefore, indicate that, unlike somatic cells, spermatozoa possess the ability to remove the 8OHdG base adduct formed following free radical attack but lack the machinery required to repair oxidative DNA lesions beyond this first step. The lack of APE1, in particular, suggests why oxidative stress can create 8OHdG lesions and a concomitant signal in the SCSA assay but fails to generate an equivalent short-term response in the TUNEL assay (Ramos and Wetzels, 2001; Smith *et al*., 2013). If such oxidatively damaged cells are cultured for a prolonged period of time (>24 h), however, then TUNEL positivity does eventually appear to generate results that correlate quite closely with the outcome of both SCSA and 8OHdG assays (Ramos and Wetzels, 2001; De Iuliis *et al*., 2009b; Smith *et al*., 2013). In keeping with these results, it has recently been shown that TUNEL positivity appears at the end of the apoptotic process as the spermatozoa are close to cell death (Koppers *et al*., 2011). This observation explains why so many TUNEL positive cells are, in fact, dead (Mitchell *et al*., 2011). Precisely, how spermatozoa become TUNEL positive with the passage of time is not known. One interesting possibility is that spermatozoa possess an endonuclease that is already integrated into the chromatin structure and becomes activated as these cells enter the perimortem, as a final act of self-destruction before their ultimate phagocytosis (Sotolongo *et al*., 2005).

Assays for measuring the various stages of DNA damage, from the initial oxidative insult that creates the 8OHdG base adduct to the fragmentation of DNA detected by the TUNEL assay as the cells approach death appear to yield a high level of inter-assay correlation (Chohan *et al*., 2006). As a result, the assays used in clinical practice should not have a material influence on the diagnostic significance of the results obtained (Gorczyca *et al*., 1993; Aravindan *et al*., 1997; Smith *et al*., 2013). The only reservation is that the 8OHdG and TUNEL flow cytometry assays are impeded by the highly compacted nature of sperm chromatin and necessitate the introduction of a chromatin decondensation step, prior to commencement of the assay in order to avoid potential artefacts, as discussed at the beginning of this review (Aitken *et al*., 2010; Mitchell *et al*., 2011). Given this plethora of excellent methods for detecting DNA damage in the male germ line, we should now consider the clinical value of this information and its role in patient management.

Biological and clinical significance of DNA damage in spermatozoa

The clinical significance of DNA damage in the male germ line has been the subject of much discussion and the source of some confusion. There is an extensive literature addressing the relationship between DNA damage in spermatozoa and fertility, defined in a variety of ways and under a variety of circumstances including natural conception (Giwercman *et al*., 2010), IVF (Simon *et al*., 2010, 2013) ICSI (Zini, 2011; Simon *et al*., 2013) and IUI (Bungum *et al*., 2007). The general conclusion from these data is that there appears to be a general relationship between DNA damage and fertility but the correlations are weak and of variable significance. The most powerful associations appear to be with natural conceptions, IUI and IVF, but weak or non-existent with ICSI. Following a recent (2008 and 2013) review of this area, the Practice Committee of the American Society for Reproductive Medicine has concluded that:

- existing data do not support a consistent relationship between abnormal DNA integrity and reproductive outcomes and
- at present, the results of sperm DNA integrity testing alone do not predict pregnancy rates achieved with IUI, IVF or ICSI. However, further research may lead to validation of the clinical utility of these tests.

Although such a conservative call for caution may appear laudable and in the best interests of a paying patient population, in reality, it amounts to a straw man fallacy that does the field a disservice. Clearly, there is no *direct* relationship between the status of DNA in a sperm nucleus and the fertilizing potential of the cell. The sperm nucleus is densely compacted, inert to the point of transcriptional silence and plays no active role in the processes of capacitation and fertilization (Aitken, 2013). We have demonstrated this directly by testing the fertilizing potential of spermatozoa *in vitro*, while subjecting these cells to increasing levels of oxidative attack (Aitken *et al*., 1998). This study revealed that the DNA in the sperm nucleus is more sensitive to oxidative damage than the mechanisms regulating sperm fertilization. Indeed, at moderate levels of oxidative stress the DNA was extensively damaged and yet the spermatozoa exhibited an enhanced capacity for fertilization as a consequence of the redox regulation of sperm capacitation. Furthermore, the entire field of paternally mediated reproductive risk is dependent on the fact that spermatozoa with damaged DNA, as a consequence of paternal age, lifestyle or inadvertent toxicant exposure, *can* still fertilize oocytes and initiate development. If there is a relationship between sperm DNA damage and the fertilizing potential of these cells, it must be indirect.

One possible mechanism for such an indirect effect would be that spermatozoa experiencing very high levels of oxidative stress not only suffer from DNA fragmentation but also exhibit collateral damage to the sperm plasma membrane as a result of extensive lipid peroxidation. The latter would then be expected to precipitate a loss of motility and a reduced competence for sperm–oocyte fusion (Aitken *et al*., 1989). In this context, there is an extensive literature linking measurements of oxidative stress and fertility both *in vitro* (du Plessis *et al*., 2010; Succu *et al*. 2011) and *in vivo* (Aitken *et al*., 1991; Sikka, 2001; Tsunoda *et al*., 2012). Such an oxidative stress model would explain why DNA damage in spermatozoa tends to be correlated with fertility in situations where the functional competence of the spermatozoa is severely tested (natural conception, IUI and IVF), whereas this association is weakened when ICSI is used to achieve fertilization and the spermatozoon is but a passenger in the insemination process (Thomson *et al*, 2011; Simon *et al*., 2013).

Thus, DNA damage in human spermatozoa is just one attribute of sperm quality and not one that will be inevitably or inextricably linked with fertility. Perfectly normal spermatozoa, in terms of both their appearance and function, may still carry DNA damage, creating a problem when it comes to selecting spermatozoa for ICSI (Avendaño and Oehninger, 2011). The significance of DNA damage in spermatozoa is not about predicting fertility but rather about its potential to modify the genetic constitution of the embryo. It is absolutely incontrovertible that DNA damage in the father's germ line can influence

embryonic development. Indeed, there is entire toxicology literature describing tests such as 'the dominant lethal assay' which are completely dependent on the way in which toxicants can influence embryogenesis by working through the father's germ line (Singer et al., 2006). Epidemiologically, the link between childhood cancer and paternal exposure to environmental or lifestyle factors must also involve a similar chain of cause-and-effect between DNA damage in spermatozoa and an increased genetic/epigenetic mutational load in the offspring (Lee et al., 2009; Milne et al., 2012; Peters et al., 2012).

The mechanism by which DNA damage in spermatozoa influences the mutational load carried by the offspring probably involves a significant degree of collusion with the oocyte. As soon as spermatozoa fertilize the oocyte, the oocyte surveys the amount of DNA damage present in the sperm chromatin and immediately launches into a round of DNA repair that precedes S-phase of the first mitotic division (Shimura et al., 2002). If the oocyte makes a mistake, or is inefficient in effecting this repair, then the potential exists to create a mutation that will be present in every cell of the body (Aitken and Krausz, 2001; Aitken et al., 2004). Such mechanisms could plausibly contribute to the increased burden of disease borne by assisted reproductive technology (ART) children (Hansen et al., 2002; Gosden et al., 2003). In addition to an inflated incidence of birth defects, infants produced by ART are also significantly more likely to be admitted to a neonatal intensive care unit, to be hospitalized and to stay in hospital longer than their naturally conceived counterparts (reviewed by Aitken and Curry, 2011). Recent studies have also shown abnormal patterns of retinal vascularization in ART children and an 8-fold increase in the incidence of undescended testicles in boys conceived by ICSI (Aitken and Curry, 2011). Although studies of the health and well-being of children conceived by ART are still in their infancy, it is already clear that there is an issue to address and the closer we look, the more problems we are likely to find.

Thanks to the DNA-repair capacity of the oocyte, the risk of overt birth defects in ART children is extremely low. Nevertheless, we cannot use this information to become complacent about the safety of ART treatments involving the use of severely damaged DNA of paternal origin (Gandini et al., 2004). In this circumstance, absence of evidence is not evidence of absence. Only a very small percentage of human DNA encodes functional genes and only a very few of those genes will generate an overt phenotype when damaged. This is why spontaneous major birth defects are so rare. However, all because a child looks phenotypically normal, we cannot conclude that he/she is not carrying harmful genetic or epigenetic mutations that will cause disease later in life or in subsequent generations (Halliday, 2012).

Conclusions

The major purpose of surveying DNA damage in spermatozoa does not entirely rest on the ability of this criterion to predict fertility. Such assays also provide important information about the underlying quality of spermatogenesis and the risk that damaged genetic material will be transmitted to the offspring. There can be no doubt that DNA damage in the paternal germ line has the potential to generate mutations in the embryo that will affect the progress of pregnancy and the health and well-being of the offspring. In this context, it is no surprise that DNA damage in spermatozoa is significantly correlated with impaired preimplantation embryo development as well an increase in the incidence of miscarriage in the ensuing pregnancy (Razavi et al., 2003; Zini and Sigman, 2009; Robinson et al., 2012). As a consequence, we should be screening male patients with appropriate DNA integrity assays as a matter of 'best practice' with the aim of providing patients with information about possible risks to their pregnancy and to trigger management strategies designed to reduce the level of DNA damage in their spermatozoa. How we achieve the latter is a key question that we shall have to address through research. We should clearly aim to reduce iatrogenic contributions to DNA damage in spermatozoa resulting from the deployment of suboptimal methods for the preparation and incubation of spermatozoa, as far as possible (Twigg et al., 1998a; Ainsworth et al., 2007). In addition, if oxidative stress is a major cause of DNA damage in the germ line, then antioxidants should be part of the cure. Given the promise offered by such therapy, it is remarkable that we still await a carefully conducted, controlled, randomized, double-blind, cross-over trial to determine whether antioxidants are effective agents in countering sperm DNA damage *in vivo*. (Gharagozloo and Aitken, 2011).

Authors' roles

R.B. and R.J.A. conceived the article and R.J.A. generated the first draft of this review. R.B., T.B.S. and G.N.D. then commented on the manuscript and all the authors approved the final version of this article.

Funding

This work was supported by National Health and Medical Research Council (program grant number 494802) Australian Research Council (project grant number DP110103951)

Conflict of interest

None declared.

References

Ainsworth C, Nixon B, Jansen RP, Aitken RJ. First recorded pregnancy and normal birth after ICSI using electrophoretically isolated spermatozoa. *Hum Reprod* 2007;**22**:197–200.

Aitken RJ. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev* 1995;**7**:659–668.

Aitken RJ. Can spermatozoa respond to changes in their redox status with the selective activation of gene transcription? *Antioxid Redox Signal* 2013; **18**:184–185.

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

Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* 2011;**14**: 367–381.

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

Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001;**122**:497–506.

Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 1989; **41**:83–197.

Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol* 1991;**164**:542–551.

Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K, Richardson DW. Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *J Reprod Fertil* 1992;**94**:451–462.

Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, Irvine S. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev* 1997;**47**:468–482.

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

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

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.

Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod* 2010;**25**:2415–2426.

Aitken RJ, Hanson AR, Kuczera L. Electrophoretic sperm isolation: optimization of electrophoresis conditions and impact on oxidative stress. *Hum Reprod* 2011;**26**:1955–1964.

Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J Biol Chem* 2012;**287**:33048–33060.

Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. *J Androl* 1987;**8**:338–348.

Aravindan GR, Bjordahl J, Jost LK, Evenson DP. Susceptibility of human sperm to *in situ* DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp Cell Res* 1997;**236**:231–237.

Avendaño C, Oehninger S. DNA fragmentation in morphologically normal spermatozoa: how much should we be concerned in the ICSI era? *J Androl* 2011;**32**:356–363.

Baker MA, Aitken RJ. The importance of redox regulated pathways in sperm cell biology. *Mol Cell Endocrinol* 2004;**216**:47–54.

Baker MA, Aitken RJ. Reactive oxygen species in spermatozoa: methods for monitoring and significance for the origins of genetic disease and infertility. *Reprod Biol Endocrinol* 2005;**3**:67.

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

Barroso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 2000;**15**:1338–1344.

Bennetts LE, Aitken RJ. A comparative study of oxidative DNA damage in mammalian spermatozoa. *Mol Reprod Dev* 2005;**71**:77–87.

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.

Boaz SM, Dominguez K, Shaman JA, Ward WS. Mouse spermatozoa contain a nuclease that is activated by pretreatment with EGTA and subsequent calcium incubation. *J Cell Biochem* 2008;**103**:1636–1645.

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.

Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* 2006;**27**:53–59.

Crow JF. The origins, patterns and implications of human spontaneous mutation. *Nat Rev Genet* 2000;**1**:40–47.

de Lamirande E, Lamothe G. Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. *Free Radic Biol Med* 2009;**46**:502–510.

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

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* 2009b;**81**:517–524.

Donà G, Fiore C, Andrisani A, Ambrosini G, Brunati A, Ragazzi E, Armanini D, Bordin L, Clari G. Evaluation of correct endogenous reactive oxygen species content for human sperm capacitation and involvement of the NADPH oxidase system. *Hum Reprod* 2011;**26**:3264–3273.

du Plessis SS, McAllister DA, Luu A, Savia J, Agarwal A, Lampiao F. Effects of H(2)O(2) exposure on human sperm motility parameters, reactive oxygen species levels and nitric oxide levels. *Andrologia* 2010;**42**:206–210.

Enciso M, Johnston SD, Gosálvez J. Differential resistance of mammalian sperm chromatin to oxidative stress as assessed by a two-tailed comet assay. *Reprod Fertil Dev* 2011;**23**:633–637.

Foresta C, Zorzi M, Rossato M, Varotto A. Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *Int J Androl* 1992;**15**:330–337.

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

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

Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, Leter G, Ciriminna R, Culasso F, Dondero F, Lenzi A *et al*. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Hum Reprod* 2004;**19**:1409–1417.

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

Giwercman A, Lindstedt L, Larsson M, Bungum M, Spano M, Levine RJ, Rylander L. Sperm chromatin structure assay as an independent predictor of fertility *in vivo*: a case-control study. *Int J Androl* 2010;**33**:e221–e227.

Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 1996;**17**:276–287.

Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z. Presence of DNA strand breaks and increased sensitivity of DNA *in situ* to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. *Exp Cell Res* 1993;**207**:202–205.

Gosden R, Trasler J, Lucifero D, Faddy M. Rare congenital disorders, imprinted genes, and assisted reproductive technology. *Lancet* 2003;**361**:1975–1977.

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* 2005;**26**:349–353.

Halliday J. Outcomes for offspring of men having ICSI for male factor infertility. *Asian J Androl* 2012;**14**:116–120.

Hansen M, Kurinczuk JJ, Bower C, Webb S. The risk of major birth defects after intracytoplasmic sperm injection and *in vitro* fertilization. *N Engl J Med* 2002;**346**:725–730.

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.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM, Jin F. Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *J Natl Cancer Inst* 1997;**8**:238–244.

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;**65**:519–524.

Koppers AJ, Mitchell LA, Wang P, Lin M, Aitken RJ. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. *Biochem J* 2011;**436**:687–698.

Leduc F, Maquennehan V, Nkoma GB, Boissonneault G. DNA damage response during chromatin remodeling in elongating spermatids of mice. *Biol Reprod* 2008;**78**:324–332.

Lee JD IV, Allen MJ, Balhorn R. Atomic force microscope analysis of chromatin volumes in human sperm with head-shape abnormalities. *Biol Reprod* 1997;**56**:42–49.

Lee KM, Ward MH, Han S, Ahn HS, Kang HJ, Choi HS, Shin HY, Koo HH, Seo JJ, Choi JE *et al.* Paternal smoking, genetic polymorphisms in CYP1A1 and childhood leukemia risk. *Leuk Res* 2009;**33**:250–258.

Li Z, Yang J, Huang H. Oxidative stress induces H2AX phosphorylation in human spermatozoa. *FEBS Lett* 2006;**580**:6161–6168.

Loft S, Kold-Jensen T, Hjollund NH, Giwercman A, Gyllemborg J, Ernst E, Olsen J, Scheike T, Poulsen HE, Bonde JP. Oxidative DNA damage in human sperm influences time to pregnancy. *Hum Reprod* 2003; **18**:1265–1272.

Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, Sakkas D. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol Reprod* 1995;**52**:864–867.

Milne E, Greenop KR, Scott RJ, Bailey HD, Attia J, Dalla-Pozza L, de Klerk NH, Armstrong BK. Parental prenatal smoking and risk of childhood acute lymphoblastic leukemia. *Am J Epidemiol* 2012;**175**:43–53.

Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *Int J Androl* 2011;**34**:2–13.

Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl* 2003;**24**:253–262.

Oger I, Da Cruz C, Panteix G, Menezo Y. Evaluating human sperm DNA integrity: relationship between 8-hydroxydeoxyguanosine quantification and the sperm chromatin structure assay. *Zygote* 2003; **11**:367–371.

Peters S, Glass DC, Reid A, de Klerk N, Armstrong BK, Kellie S, Ashton LJ, Milne E, Fritschi L. Parental occupational exposure to engine exhausts and childhood brain tumors. *Int J Cancer* 2012 (in press).

Pujianto DA, Curry BJ, Aitken RJ. Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. *Endocrinology* 2010;**151**:1269–1279.

Ramos L, Wetzels AM. Low rates of DNA fragmentation in selected motile human spermatozoa assessed by the TUNEL assay. *Hum Reprod* 2001;**16**:1703–1707.

Razavi S, Nasr-Esfahani MH, Madani M, Mafi A, Moghdam A. Effect of human sperm chromatin anomalies on fertilization outcome post-ICSI. *Andrologia* 2003;**35**:238–243.

Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-Brown J, Coomarasamy A. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod* 2012;**27**:2908–2917.

Sailer BL, Jost LK, Evenson DP. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J Androl* 1995;**16**:80–87.

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

Sawyer DE, Mercer BG, Wiklendt AM, Aitken RJ. Quantitative analysis of gene-specific DNA damage in human spermatozoa. *Mutat Res* 2003; **529**:21–34.

Shen HM, Chia SE, Ong CN. Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *J Androl* 1999; **20**:718–23.

Shimura T, Inoue M, Taga M, Shiraishi K, Uematsu N, Takei N, Yuan ZM, Shinohara T, Niwa O. p53-dependent S-phase damage checkpoint and pronuclear cross talk in mouse zygotes with X-irradiated sperm. *Mol Cell Biol* 2002;**22**:2220–2228.

Sierens J, Hartley JA, Campbell MJ, Leathem AJ, Woodside JV. *In vitro* isoflavone supplementation reduces hydrogen peroxide-induced DNA damage in sperm. *Teratog Carcinog Mutagen* 2002;**22**:227–234.

Sikka SC. Relative impact of oxidative stress on male reproductive function. *Curr Med Chem* 2001;**8**:851–862.

Simon L, Brunborg G, Stevenson M, Lutton D, McManus J, Lewis SE. Clinical significance of sperm DNA damage in assisted reproduction outcome. *Hum Reprod* 2010;**25**:1594–1608.

Simon L, Castillo J, Oliva R, Lewis SE. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. *Reprod Biomed Online* 2011a;**23**:724–734.

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

Simon L, Proutski I, Stevenson M, Jennings D, McManus J, Lutton D, Lewis SE. Sperm DNA damage has a negative association with live-birth rates after IVF. *Reprod Biomed Online* 2013;**26**:68–78.

Singer TM, Lambert IB, Williams A, Douglas GR, Yauk CL. Detection of induced male germline mutation: correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. *Mutat Res* 2006;**598**:164–193.

Singh NP, Danner DB, Tice RR, McCoy MT, Collins GD, Schneider EL. Abundant alkali-sensitive sites in DNA of human and mouse sperm. *Exp Cell Res* 1989;**184**:461–470.

Smith R, Vantman D, Ponce J, Escobar J, Lissi E. Total antioxidant capacity of human seminal plasma. *Hum Reprod* 1996;**11**:1655–1660.

Smith TB, Dun MD, Smith ND, Curry BJ, Connaughton HS, Aitken RJ. The presence of a truncated base excision repair pathway in human spermatozoa, mediated by OGG1. *J Cell Sci* 2013 (in press).

Sotolongo B, Huang TT, Isenberger E, Ward WS. An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J Androl* 2005;**26**:272–280.

Succu S, Berlinguer F, Pasciu V, Satta V, Leoni GG, Naitana S. Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. *J Pineal Res* 2011;**50**:310–318.

Thomson LK, Zieschang JA, Clark AM. Oxidative deoxyribonucleic acid damage in sperm has a negative impact on clinical pregnancy rate in intrauterine insemination but not intracytoplasmic sperm injection cycles. *Fertil Steril* 2011;**96**:843–847.

Tsunoda S, Kawano N, Miyado K, Kimura N, Fujii J. Impaired fertilizing ability of superoxide dismutase 1-deficient mouse sperm during *in vitro* fertilization. *Biol Reprod* 2012;**87**:121.

Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol Hum Reprod* 1998a;**4**:439–445.

Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol Hum Reprod* 1998b; **4**:439–445.

Villani P, Eleuteri P, Grollino MG, Rescia M, Altavista P, Spanò M, Pacchierotti F, Cordelli E. Sperm DNA fragmentation induced by DNAse I and hydrogen peroxide: an *in vitro* comparative study among different mammalian species. *Reproduction* 2010;**140**:445–452.

Wang X, Sharma RK, Sikka SC, Thomas AJ Jr, Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril* 2003;**80**:531–535.

Ward WS. Function of sperm chromatin structural elements in fertilization and development. *Mol Hum Reprod* 2010;**16**:30–36.

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

Zini A. Are sperm chromatin and DNA defects relevant in the clinic? *Syst Biol Reprod Med* 2011;**57**:78–85.

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