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NEW RESEARCH HORIZON ***Review***

On the possible origins of DNA damage in human spermatozoa

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ABSTRACT: DNA damage in the male germ line has been linked with a variety of adverse clinical outcomes including impaired fertility, an increased incidence of miscarriage and an enhanced risk of disease in the offspring. The origins of this DNA damage could, in principle, involve: (i) abortive apoptosis initiated post meiotically when the ability to drive this process to completion is in decline (ii) unresolved strand breaks created during spermiogenesis to relieve the torsional stresses associated with chromatin remodelling and (iii) oxidative stress. In this article, we present a two-step hypothesis for the origins of DNA damage in human spermatozoa that highlights the significance of oxidative stress acting on vulnerable, poorly protaminated cells generated as a result of defective spermiogenesis. We further propose that these defective cells are characterized by several hallmarks of 'dysmaturity' including the retention of excess residual cytoplasm, persistent nuclear histones, poor zona binding and disrupted chaperone content. The oxidative stress experienced by these cells may originate from infiltrating leukocytes or, possibly, the entry of spermatozoa into an apoptosis-like cascade characterized by the mitochondrial generation of reactive oxygen species. This oxidative stress may be exacerbated by a decline in local antioxidant protection, particularly during epididymal maturation. Finally, if oxidative stress is a major cause of sperm DNA damage then antioxidants should have an important therapeutic role to play in the clinical management of male infertility. Carefully controlled studies are now needed to critically examine this possibility.

Key words: DNA damage / sperm / apoptosis / reactive oxygen species / chromatin

Introduction

DNA damage in the male germ line is a major contributor to infertility, miscarriage and birth defects in the offspring (reviewed by Aitken *et al*., 2009). The data to support such a statement comes primarily from the animal literature, which is unequivocal in demonstrating that the genetic integrity of the male germ line plays a major role in determining the normality of embryonic development. Thus the dominant lethal assay, one of the major weapons in the toxicologist's armamentarium is based on the fundamental principle that exposure of male rats to a given toxin can impede the progress of any ensuing pregnancies, leading to high rates of embryonic resorption (Adler, 2000). The toxicology literature abounds with examples of compounds that are active in the dominant lethal assay including 1,3-butadiene, diepoxybutane, ethylene thiourea, acrylamide and a wide variety of anticancer drugs (Dearfield, 1994; Anderson *et al.*, 1996; Witt and Bishop, 1996; Tyla *et al*., 2000; Anderson, 2001). Extended dominant lethal assays have also been advocated that involve mating treated males to untreated females and examining the latter 1 day before term in order to search for congenital malformations such as cleft palate or hydrocephaly (Anderson, 2005). The offspring can also be allowed to develop to adulthood so that susceptibility to tumours or behavioural defects can be studied. Using this kind of approach the genotoxic consequences of chronically exposing male rodents to cyclophosphamide and, in the case of mice, 1,3-butadiene, have again been confirmed in terms of dominant lethal mutations and congenital malformations in the offspring. In a more recent study, mouse spermatozoa suffering from DNA damage following a freeze-thaw cycle in the absence of cryoprotectant, were injected into mouse oocytes to determine whether the use of such damaged gametes had any impact on the progress of pregnancy and/or the health and wellbeing of the progeny (Fernández-Gonzalez *et al*., 2008). The results of this important study were to demonstrate powerful adverse effects on embryo development and the behaviour, post-natal growth and longevity of the offspring, as well as their susceptibility to tumours. Similar studies involving the use of intra-cytoplasmic sperm injection (ICSI) to transfer spermatozoa subjected to medium containing putative endonucleases has also demonstrated a significant impairment of embryo implantation rate (Pérez-Crespo *et al*., 2008).

Given the wealth of existing animal data, it must be incontrovertible that DNA damage in the male germ line has the potential to disrupt the viability and developmental normality of human pregnancies. In support of this concept, reports have appeared suggesting causative linkages between paternal smoking, oxidative damage to sperm DNA and the incidence of cancer in children (Fraga *et al*., 1996; Ji *et al*., 1997; Chang, 2009). Furthermore, a great deal of correlative

data has been presented in recent years suggesting that DNA damage in human spermatozoa is associated with the impairment of oocyte fertilization, the preimplantation development of the embryo and subsequent progress of pregnancy to term (reviewed Aitken *et al.*, 2009; Zini and Sigman, 2009). However, these adverse outcomes are not consistently observed across all studies (Zini and Sigman, 2009), generating some scepticism about the clinical significance of such damage.

This inconsistency should not generate complacency amongst infertility specialists for a number of reasons. Firstly, DNA damage in spermatozoa is but one factor among many that will ultimately determine the outcome of a given pregnancy. In the case of DNA damage to the male germ line, much will depend on the type of damage induced, when it was induced, the region of the genome affected and the ability of the embryo to repair the damage before initiation of the S-phase that precedes the first mitotic division (Aitken *et al.*, 2004, 2009). Even if the embryo is not completely effective in repairing the genetic damage brought into the zygote by the fertilizing spermatozoon, the chances that a phenotype will be generated in the F1 generation are highly remote. For example, dominant genetic conditions such as achondroplasia reflect the genetic deterioration of male germ cells as a consequence of ageing (Crow, 2000). Although DNA damage is quite prevalent in the spermatozoa of ageing males (Singh *et al.*, 2003; Schmid *et al.*, 2007), this damage is only associated with a phenotypic change in the offspring in <1 case per 100 000. Thus, the fact that normal babies can be born as a result of using DNA-damaged gametes in assisted conception cycles (Gandini *et al.*, 2004) does not mean necessarily that these children are genetically or epigenetically normal or that this damage will not generate a phenotypic change in future generations as a consequence of such mechanisms as double recessive gene expression, haploid insufficiency or, in the case of X chromosome mutations, the future birth of male offspring. It is also possible that whatever defects are present in the offspring as a result of fertilization with DNA damaged spermatozoa cannot be recognized at birth. A case in point is the recent finding that DNA damage in the spermatozoa of ageing males is associated with the appearance of complex polygenic neurological conditions in the progeny including epilepsy, spontaneous schizophrenia, autism and bipolar disease (Sipos *et al.*, 2004; Reichenberg *et al.*, 2006; Aitken and De Iuliis, 2007a, b; Frans *et al.*, 2008). In light of such considerations, DNA damage in the germ line should be regarded as a potential risk factor for the development of normal human embryos that must be addressed in the name of 'best practice', if for no other reason. In this context, two questions are critical: how does the DNA damage arise and how should such damage be clinically managed?

Origins of DNA damage in spermatozoa

To date, three major mechanisms for the creation of DNA damage in the male germ line have been proposed involving chromatin remodelling by topoisomerase, oxidative stress and abortive apoptosis. It should be recognized however, that these proposed mechanisms are not mutually exclusive and, in reality, DNA damage may arise from combinations of all three mechanisms.

Chromatin remodelling and DNA strand breaks

One of the first hypotheses to be advanced concerning the origins of DNA damage in the male germ line, focused on the physiological strand breaks created by topoisomerase during spermiogenesis as a means of relieving the torsional stresses created as DNA is condensed and packaged into the differentiating sperm head (Sakkas *et al.*, 1999; Marcon and Boissonneault, 2004). Normally these strand breaks are marked by a histone phosphorylation event (gamma-H2AX; H2A histone family, member X) and fully resolved by topoisomerase before spermatozoa are released from the germinal epithelium during spermiogenesis (Leduc *et al.*, 2008). Should the testes be subjected to a genotoxic stress of some kind, such as mild testicular heating or gamma irradiation, then the DNA damage induced is reflected by an increased incidence of gamma H2AX-positive foci in precursor germ cells as the DNA strand breaks are marked for repair (Hamer *et al.*, 2003; Forand *et al.*, 2004; Paul *et al.*, 2008). If this repair process is impaired for some reason then the expected outcome might be the existence of spermatozoa possessing high levels of DNA damage associated with the persistent expression of gamma-H2AX. To the author's knowledge such persistent gamma-H2AX expression has not been reported to date for human spermatozoa exhibiting high levels of DNA damage. However, a recent publication suggested that mature ejaculated human spermatozoa retain the H2AX phosphorylation machinery, presumably as a means of marking DNA strand breaks for subsequent repair in the oocyte (Li *et al.*, 2008). In this study, treatment of human spermatozoa with adriamycin resulted in the creation of double strand breaks and the concomitant expression of gamma-H2AX along with DNA maintenance/repair proteins RAD50 and 53BP1 (Li *et al.*, 2008). Similar results have also been reported by the same group in spermatozoa exposed to oxidative stress, such that when human spermatozoa were exposed to hydrogen peroxide then H2AX phosphorylation was induced in a time- and dose-dependent manner (Li *et al.*, 2006). That a transcriptionally and translationally silent spermatozoon with such tightly compacted, histone-depleted chromatin, possesses the capacity to detect and mark DNA strand breaks for repair by phosphorylating H2AX is fascinating and deserves further attention. At face value such a concept runs contrary to the widely held belief that the chromatin with these cells is inert and once damaged has to wait until fertilization for repair to be effected by the embryo during a post fertilization round of DNA repair that unequivocally does involve activation of the gamma-H2AX signalling pathway (Aitken *et al.*, 2004; Derijck *et al.*, 2006).

Oxidative stress

One of the major contributory factors to defective sperm function is oxidative stress. This was first recognized by Thaddeus Mann and his colleagues at the University of Cambridge more than 30 years ago. In a series of landmark articles, this group established the vulnerability of mammalian spermatozoa to free radical attack and the induction of a lipid peroxidation process that disrupts the integrity of the plasma membrane and impairs sperm motility (Jones *et al.*, 1978, 1979). This susceptibility stems from the presence of targets for free radical attack in these cells including a superabundance of polyunsaturated fatty acids. The presence of unsaturated fatty acids in the plasma membrane is necessary to create the membrane fluidity required by the membrane fusion events associated with fertilization, particularly acrosomal exocytosis and fusion with the oolemma. Thus as much

as 50% of the fatty acid in a human spermatozoon is docosahexaenoic acid with six double bonds per molecule (Jones *et al*., 1979). Unfortunately, such highly unsaturated fatty acids are particularly prone to oxidative attack because the conjugated nature of the double bonds facilitates such processes as hydrogen abstraction, which initiates the lipid peroxidation cascade. The latter can be promoted by the presence of transition metals such as iron and copper that can vary their valency state by gaining or losing electrons. Significantly, there is sufficient free iron and copper in human seminal plasma to promote lipid peroxidation once this process has been initiated (Kwenang *et al*., 1987). Such transition metals can also promote the ability of reactive oxygen species (ROS) to attack another important substrate in mammalian spermatozoa—the DNA present in the sperm nucleus and mitochondria.

Mitochondrial DNA is particularly vulnerable to free radical attack because it is essentially unprotected (Sawyer *et al*., 2001). This vulnerability makes mitochondrial DNA a particularly sensitive marker for monitoring oxidative stress in the male germ line. However, since this DNA makes no contribution to the functionality of the spermatozoon or the subsequent development of the embryo, such damage has little biological meaning. Sperm nuclear DNA, on the other hand, is much harder to damage because it is tightly compacted with protamines that, in Eutherian mammals at least, are further stabilized by the creation of inter- and intra-molecular disulphide bonds (Sawyer *et al*., 2003; Bennetts and Aitken, 2005). Nevertheless, free radicals can still attack this material, engaging in H-abstraction reactions with the ribose unit and inducing the formation of DNA base adducts. Both of these processes greatly destabilize the DNA structure and may ultimately result in the formation of DNA strand breaks. It has been known for some time that the spermatozoa of subfertile patients contain particularly high levels of 8-hydroxy-2′-deoxyguanosine (8OHdG), the major oxidized base adduct formed when DNA is subjected to attack by ROS (Kodama *et al*., 1997). We have recently not only confirmed this observation but also found the presence of 8OHdG adducts in human spermatozoa to be highly correlated with DNA strand breaks, as assessed with a TUNEL assay. Indeed the correlation between DNA strand breaks and 8OHdG formation is so strong ($R = 0.756$; Fig. 1) that it would reasonable to conclude that oxidative stress is one of the major contributors to DNA damage in the male germ line. Moreover, this conclusion is not only valid for the cohort of unselected donors used for this analysis but also for the cohort of males attending assisted conception clinics (De Iuliis *et al*., 2009).

The source of the oxidative stress responsible for creating DNA damage in the germ line could theoretically involve a number of factors including: (i) a loss of antioxidant protection in the male reproductive tract, (ii) infection (iii) xenobiotic exposure (iv) intrinsic radical production by spermatozoa, as discussed below.

Loss of antioxidant protection

Antioxidant protection is particularly critical for spermatozoa because these cells are relatively deficient in ROS-scavenging enzymes as a consequence of the limited volume, and restricted distribution, of cytosolic space. As a result, these cells are particularly dependent on the antioxidant protection offered by the male reproductive tract. This is of major importance in the epididymis where spermatozoa will spend several days completing the first stages of their post-testicular maturation. In order to protect the spermatozoa during their sojourn in the epididymis this organ secretes a complex array of antioxidant factors into the lumen of the epididymal tubules including small molecular mass free radical scavengers (vitamin C, uric acid, taurine, thioredoxin) and highly specialized extracellular antioxidant enzymes, including unique isoforms of superoxide dismutase and glutathione peroxidase, particularly glutathione peroxidase 5 (GPx5) (Vernet *et al*., 1996). GPx5 is an unusual glutathione peroxidase in that it is solely expressed in the caput epididymis under androgenic control. It is also unusual in that it lacks a selenocysteine residue while still retaining its antioxidant properties (Vernet *et al*., 1996, 2004). This protein associates with the sperm surface during epididymal transit and protects the spermatozoa from peroxide mediated attack as they are undergoing maturation (Vernet *et al*., 1996;

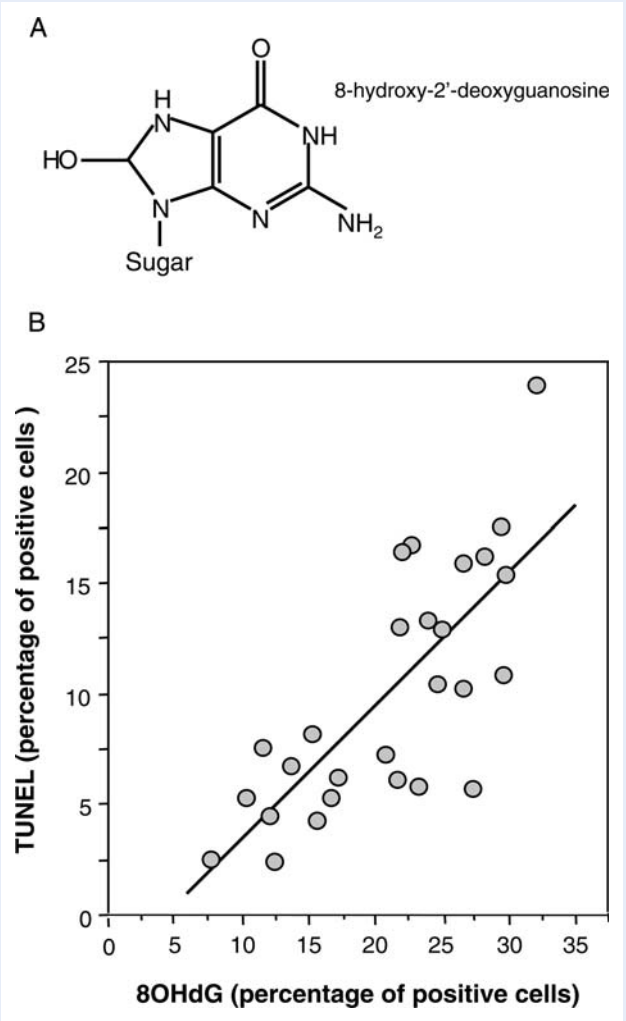

Figure 1 A significant proportion of DNA damage in the male germ line is associated with oxidative stress.

(**A**) 8-OH, 2′-deoxyguanosine (8OHdG) is marker for oxidative damage to DNA. (**B**) DNA damage in human spermatozoa measured with a TUNEL assay is highly correlated with the formation of oxidative DNA base adducts. Results presented as the percentage of positive cells according to flow cytometry ($R = 0.756$, $P < 0.001$).

Drevet, 2006). The functional significance of this molecule has recently been demonstrated with publication of the phenotype of the GPx5 knockout mouse (Chabory *et al*., 2009). This mouse exhibits an age-dependent increase in oxidative damage to sperm DNA which is, in turn, associated with high rates of miscarriage in mated females as well as birth defects in the offspring.

Whether defects in the antioxidant protection afforded by seminal plasma is as important as the contribution made by epididymal plasma is uncertain. Unlike the epididymis, sperm spend very little time in seminal plasma. Nevertheless, the animal data tell us that the secondary sexual glands are essential for reproductive success. If these glands are surgically removed then the animals exhibit high levels of oxidative DNA damage to their spermatozoa and the development of the embryos is impaired, leading to physical and behavioural defects in the offspring (O *et al*., 2006; Wong *et al*., 2007). In the case of human seminal plasma, the antioxidant capacity of this fluid can provide an important insight into the level of oxidative stress a given subject might be under, as in the low antioxidant capacity recorded for male smokers (Fraga *et al*., 1996). In non-smoking males there is also some data to suggest that DNA damage in spermatozoa is associated with a reduction in the antioxidant capacity of human semen as reflected in the levels of, for example, vitamin C (Song *et al*., 2006) carnitine (De Rosa *et al*., 2005) and co-enzyme Q_{10} (Mancini *et al*., 2005). Similarly, the total antioxidant capacity of human semen has been measured and been shown to be negatively associated with oxidative stress and fertility status (Pasqualotto *et al*., 2008a, b; Mahfouz *et al*., 2009). Although seminal plasma is richly endowed with antioxidants that can, *in vitro*, protect spermatozoa from oxidative stress and DNA damage (Twigg *et al*., 1998; Potts *et al*., 2000) whether it plays a major role *in vivo* is open to debate. Many authors have observed that as ROS generation in semen goes up, seminal antioxidant capacity goes down (Song *et al*., 2006; Pasqualotto *et al*., 2008a, b; Tremellen, 2008). It could be argued that the oxidative stress in the ejaculate was generated by the decline in antioxidant protection. However, it is just as likely that any reduction in the antioxidant status of human seminal plasma is a consequence of oxidative stress, not its cause. In other words ROS production in the ejaculate consumes antioxidant equivalents from seminal plasma lowering the level of protection that can be afforded to the viable cells in the ejaculate. In this context, the major culprits responsible for lowering the antioxidant capacity of human semen are not the spermatozoa but infiltrating leucocytes (Aitken and Baker, 1995; Sharma *et al*., 2001).

Infection and leukocytic infiltration

Every human semen sample contains leukocytes, particularly neutrophils and macrophages. Because these cells are professional generators of ROS they can make a very significant contribution to the overall levels of ROS in human semen (Aitken and West, 1990; Fig. 2A). Indeed, if human semen is simply diluted to remove its antioxidant activity and then cellular ROS generation is measured, a highly significant correlation with seminal leukocyte concentrations is observed (Fig. 2B), reflecting the fact that on a cell-by-cell basis, leukocytes are log orders of magnitude more active in the generation of ROS than spermatozoa (Aitken *et al*., 1995a, b). Since the leukocytes are sometimes entering the seminal fluids in an activated, free radical-generating state, they are potentially capable of inducing oxidative damage in the spermatozoa. Whether this is the case depends on a number of factors such as: (i) the number and sub-type of leukocytes involved, (ii) when, where and how they were activated and (iii) how efficient the male reproductive tract fluids were in protecting the spermatozoa from oxidative stress. In as much as infection is the major cause of leukocytic infiltration into the male tract, in a vast majority of cases the first time the spermatozoa will come into contact with the leukocytes should be at ejaculation. At this moment, the spermatozoa should be well protected by the antioxidants present in seminal plasma. As a result, leukocytic

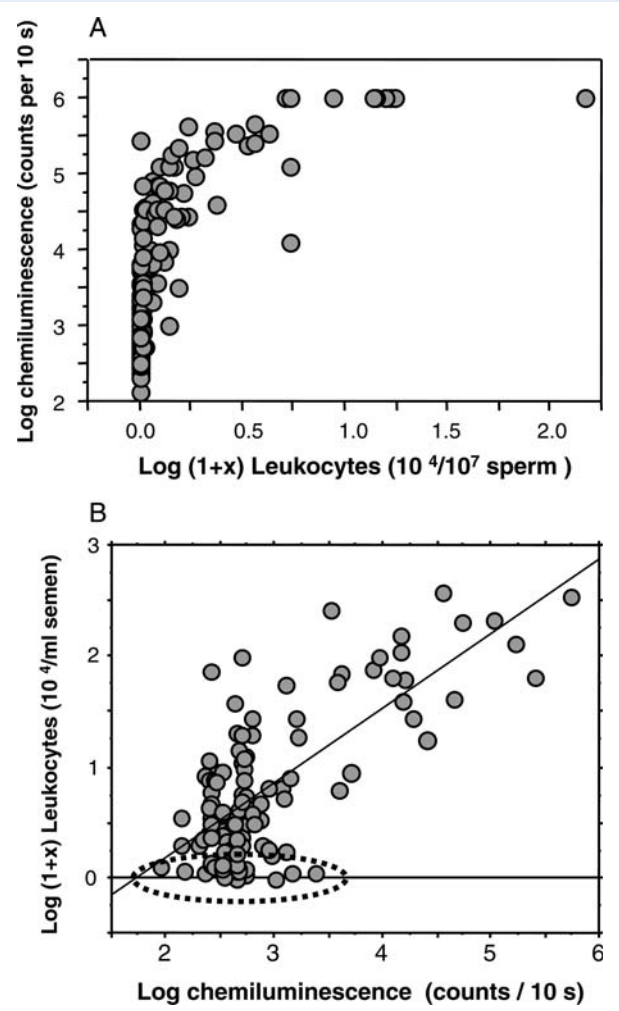

Figure 2 (**A**) Plot of leukocyte concentration against phorbol myristate acetate (PMA)-induced, luminol-peroxidase mediated chemiluminescence. Note the chemiluminescence signal generated by these Percoll-washed sperm populations varies over log orders of magnitude in the absence of leukocyte contamination. (**B**) Positive correlation between the luminol signals generated in unfractionated human semen and leukocyte (CD45 positive) contamination (Aitken *et al*., 1995a, b). However, encircled area indicates that luminol-dependent chemiluminescence can vary by a log order of magnitude in the absence of detectable leukocyte contamination, emphasizing the underlying contribution of spermatozoa to the luminol signals obtained.

infiltration into the ejaculate, even to the point of leukocytospermia, should have little impact on the functionality of the spermatozoa or the levels of DNA damage in their nuclei (Aitken *et al*., 1995a, b; Henkel *et al*., 2003; Moskovtsev *et al*., 2007). However, where the infection is chronic (Kullisaar *et al*., 2008) or where it is epididymal in origin (Haidl *et al*., 2008), then a state of oxidative stress can arise which is associated with the induction of significant DNA damage (Alvarez *et al*., 2002).

Leucocytes may also be instrumental in creating iatrogenic sperm DNA damage in assisted conception cycles, when the protective action of seminal plasma is removed and the spermatozoa are inadvertently co-cultured with contaminating leukocytes in media that may contain catalytic amounts of transition metals (Gomez and Aitken, 1996). Under these circumstances, there is every possibility that leukocyte derived ROS will impede oocyte fertilization and development. Indeed a good prediction of *in vitro* fertilization success has been secured using sperm morphology and leukocyte contamination (measured with an *N*-formyl-methionyl-leucyl-phenylalanine provocation test) as the only independent variables in a multiple regression equation (Sukcharoen *et al*., 1995).

Exposure to redox-cycling compounds

Oxidative stress and DNA damage could also be induced in the male germ line by xenobiotics that either redox cycle or activate free radical production by the spermatozoa. At present, there is very little information available on the impact of xenobiotics on free radical generation and oxidative DNA damage in spermatozoa. Recent analyses of the impact of quinones and catechol estrogens on free radical production by human spermatozoa indicated that these cells have the one electron reduction/oxidation machinery needed to activate such compounds and initiate ROS generation (Bennetts *et al*., 2008; Hughes *et al*., 2009). Moreover, these studies suggest that such redox cycling activity is perfectly capable of inducing significant DNA damage *in vitro*. Whether patients come into contact with such compounds in sufficient quantities to induce oxidative DNA damage in the germ line, is not known at the present time. Preliminary studies are certainly suggestive (Bonde *et al*., 2008) particularly in the context of chemotherapy (Barton *et al*., 2007) but much more extensive analyses are required before general conclusions can be drawn.

ROS production by spermatozoa

Spermatozoa were the first cell type in which ROS generation was detected when Tosic and Walton (1946) reported the production of hydrogen peroxide by bovine spermatozoa in Nature, more than half a century ago. Since that time, the production of ROS has been reported for every Eutherian mammal examined including man (Aitken and Clarkson, 1987), rabbit (Holland *et al*., 1982), mouse (Alvarez and Storey, 1984), rat (Vernet *et al*., 2001) boar (Guthrie and Welch, 2006) and stallion (Ball *et al*., 2001). Within these cells there can be little doubt that the major source of ROS is mitochondrial (Chapman *et al*., 1985; Koppers *et al*., 2008) although elements of an NADPH oxidase complex may also be present (Aitken *et al*., 1997; Shukla *et al*., 2005; Córdoba *et al*., 2006; Sabeur and Ball, 2007). Excessive mitochondrial production of ROS is highly correlated with the suppression of human sperm motility (Koppers *et al*., 2008) and is probably involved in the induction of DNA damage, although conclusive evidence on this point is currently lacking. The fact that 8OHdG formation in human spermatozoa is negatively associated with mitochondria membrane potential is certainly suggestive (De Iuliis *et al*., 2009).

Oxidative damage and chromatin remodelling

The induction of DNA damage may involve more than just the generation of ROS. An important element on the oxidative stress equation is also the susceptibility of the sperm DNA to damage. Chromomycin (CMA3) has been widely used as a reagent for assessing the efficiency of chromatin remodelling during spermiogenesis. This compound competes with protamines for binding sites in the minor groove of GC-rich DNA, so that the more deficient the protamination, the greater the degree CMA3 fluorescence. Using this probe we have found a very tight relationship between the efficiency of sperm chromatin protamination and the degree of oxidative DNA damage (De Iuliis *et al*., 2009). This finding is in keeping with a number of studies implicating poor chromatin remodelling in the origins of DNA damage in human spermatozoa (Bianchi *et al*., 1993; Aoki *et al*., 2006; Zini *et al*., 2007). Moreover, these findings corroborate independent clinical data revealing that both fertilization rate and preimplantation embryonic development are negatively correlated with CMA3 fluorescence in populations of human spermatozoa (Sakkas *et al*., 1998).

In light of these data we have articulated a two-step hypothesis for the origins of DNA damage in the germ line (Aitken *et al*., 2009; Fig. 3). According to this hypothesis the first step in the DNA damage cascade has its origins in spermiogenesis when the DNA is being remodelled prior to condensation. Defects in the chromatin remodelling process result in the production of spermatozoa that are characterized by an overall reduction in the efficiency of protamination, an abnormal protamine 1 to protamine 2 ratio and relatively high nucleohistone content (Sakkas *et al*., 1998; Carrell *et al*., 2008; De Iuliis *et al*., 2009). These defects in the chromatin remodelling process create a state of vulnerability, whereby the spermatozoa become susceptible to oxidative damage. In the second step of this DNA damage cascade, the chromatin is attacked by ROS (Fig. 3).

The ROS mediating such an attack could, as indicated above, result from impaired antioxidant defenses, redox cycling xenobiotics, or free radicals generated by infiltrating leukocytes. However, a central tenet of our hypothesis is that, in a majority of cases, the ROS that attack the DNA come from the spermatozoa themselves and, specifically, their mitochondria (Fig. 3). These poorly remodelled, vulnerable cells bear many of the hallmarks of cellular immaturity, particularly the retention of excess residual cytoplasm resulting in elevated cellular levels of several biochemical markers for the cytoplasmic space including creatine kinase, glucose-6-phosphate dehydrogenase, superoxide dismutase and lactic acid dehydrogenase (Huszar *et al*., 1988, 1990; Casano *et al*., 1991; Aitken *et al*., 1994, 1996a; Gomez *et al*., 1996). In addition, we anticipate that such immature cells will possess abnormal levels of the chaperone HSP2A, impaired zona binding abilities, poor protamination and a high cellular content of unsaturated fatty acids (Ollero *et al*., 2001; Zini *et al*., 2007; Sati *et al*., 2008). Given the importance of sperm mitochondria as a

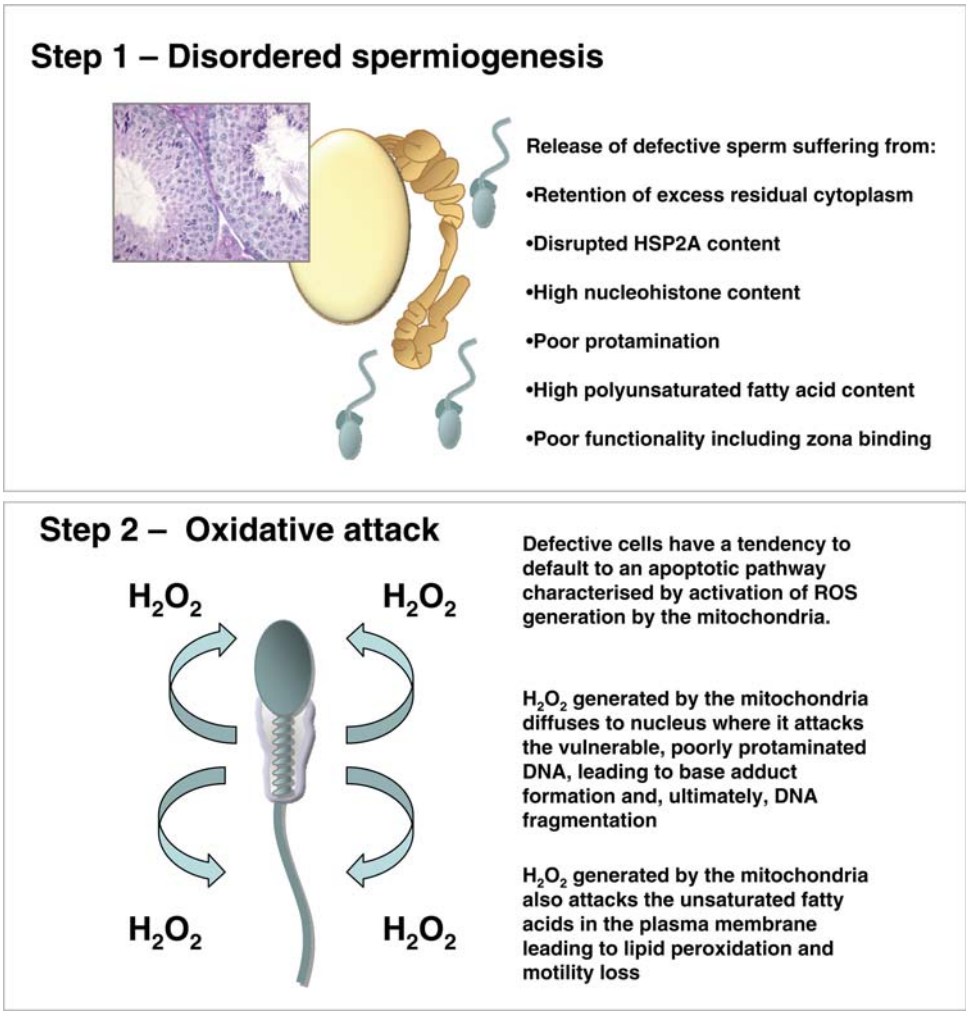

Figure 3 A two-step hypothesis for the origins of DNA damage in human spermatozoa.

In Step 1 a disruption of spermiogenesis generates defective sperm cells characterized by vulnerable chromatin. In Step 2, this vulnerable DNA is attacked by ROS leading to the formation of oxidized DNA base adducts and strand breaks. Although the ROS that mediate this attack could come from external sources such as activated leukocytes, we propose that the most common source of oxidative attack is in the form of H_2O_2 released from the spermatozoa's own mitochondria. This could be part of a process of controlled senescence similar to apoptosis. HSP2A, heat shock protein 2A diaperone.

source of ROS (Koppers *et al*., 2008) we also propose that the oxidative stress that damages sperm DNA is created by their mitochondria. The mechanisms responsible for the activation of mitochondrial ROS generation are unknown. However, we speculate that one of the contributors to this activity might be the instigation of a limited version of the intrinsic apoptotic cascade involving the activation of mitochondrial ROS generation (see below). These functionally defective, vulnerable, free radical-generating, DNA-damaged, apoptotic cells exhibiting cytoplasmic retention and a high polyunsaturated fatty acid content probably correspond to the 'immature' cells described by Huszar's group (Huszar *et al*., 1990; Sati *et al*., 2008). Unfortunately the term 'immature' is traditionally used to describe normal spermatozoa that have left the testes but are yet to complete their maturation in the epididymis. A better term for these cells might be 'dysmature' indicating an unspecified disruption in the maturation of these cells during spermiogenesis.

A final thought with respect to the mechanisms generating DNA damage is that although oxidative damage alone will create DNA strand breaks, it is also possible that the strong relationship between oxidative stress and DNA damage is indirect. Thus oxidative stress may serve to activate an endonuclease, which then induces the strands breaks associated with this process. In somatic cells it is perfectly possible for endonucleases released from the mitochondria (such as endonuclease G) or activated in the cytosol (caspase-activated deoxyribonuclease) to move into the nucleus during apoptosis and 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. Nonetheless, an alternative possibility is that the sperm chromatin contains endogenous nucleases that can be activated by stress. Recent evidence for such a chromatin-associated nuclease has been secured (Boaz *et al*., 2008). It may also be significant

that human sperm chromatin contains at east two different forms of topoisomerase, which appear to exhibit features that distinguish them from the somatic isoforms, in terms of their molecular masses and DNA decatenation activities (Har-Vardi *et al*., 2007). Whether topoisomerase inhibitors such as etoposide or camptothecin could prevent the DNA damage triggered by oxidative stress would be very interesting to know.

Sperm apoptosis

Central to this model of DNA damage in spermatozoa is the tenuous proposal that spermatozoa have to undergo a regulated form of cell death with similarities (but also differences) to the intrinsic apoptotic cascade. Spermatozoa must be capable of controlled senescence because following insemination there is a massive infiltration of leukocytes (largely neutrophils and macrophages) into the lower female tract in order to phagocytose the millions of moribund, senescent spermatozoa that did not progress to the site of fertilization. Clearly, this phagocytosis has to be silent. Thus it is vital that the phagocytes removing these cells do not generate an oxidative burst or produce pro-inflammatory cytokines that would otherwise generate a full-blown inflammatory response in response to insemination. There are many examples of silent phagocytosis in the literature and a common feature is the expression of apoptotic markers, such as phosphatidylserine, on the surface of the phagocytosed cell (Kurosaka *et al*., 2003).

A thorough analysis of this apoptotic process in spermatozoa is beyond the scope of this brief review. In essence, this process must be different from somatic cell apoptosis for a number of reasons including (i) these cells are transcritionally and translationally silent and so cannot undergo programmed cell death in the conventional sense, 'regulated cell death' might be a more appropriate term (ii) the chromatin has a reduced nucleosome content due to extensive protamination and so cannot exhibit the characteristic DNA laddering seen in somatic cells (iii) as discussed above, the physical architecture of these cells prevents endonucleases activated in the cytoplasm or released from the mitochondria from physically accessing the DNA. That said spermatozoa are capable of exhibiting some of the hallmarks of apoptosis including caspase activation and phosphatidylserine exposure on the surface of the cell (Weng *et al*., 2002). Another element of this process which appears to be functional is the generation of ROS by sperm mitochondria (Koppers *et al*., 2008; R.J. Aitken, unpublished observations). As indicated in Fig. 3, we propose that this source of ROS could make a significant contribution to the induction of oxidative stress and DNA damage in spermatozoa. Although recent studies support this hyopothesis (De Iuliis *et al*., 2009), additional studies are clearly needed to validate this concept.

Clinical management

The prominent role suggested for free radicals in the stimulation of DNA damage in spermatozoa, carries with it implications for the clinical management of patents for whom oxidative stress is a factor in the aetiology of their infertility. Below, we consider these implications in terms of the management strategies that could be followed *in vitro* and *in vivo*.

Combating oxidative stress *in vitro*

In an *in vitro* fertilization setting it is probable that contaminating leukocytes have a much greater impact on the functionality of viable human spermatozoa than dead or moribund spermatozoa (Plante *et al*., 1994). In this context, the oxidative stress created by the presence of activated leukocytes could be neutralized using a number of different strategies.

Leucocytes removal

Firstly, contaminating leukocytes could be selectively and efficiently removed from human sperm suspensions using magnetic beads or ferrofluids coated with an antibody against the common leukocyte antigen-CD45 (Aitken *et al*., 1996b). Depleting human sperm populations of leukocytes in this manner has been found to significantly enhance their capacity for fertilization (Aitken *et al*., 1996b).

Limit exposure to transition metals

Secondly, the culture medium in which the *in vitro* fertilization is conducted could be carefully selected to avoid the presence of transition metals that would otherwise only serve to stimulate the ROS-mediated damage (Gomez and Aitken, 1996).

Antioxidant supplementation

Thirdly, the culture medium could also be supplemented with antioxidants to scavenge any ROS that are generated by the leukocytes before they have an opportunity to interact with the surface of the spermatozoa. Experimental studies involving the co-culture of human spermatozoa with activated neutrophils have demonstrated the effectiveness of reduced glutathione, *N*-acetylcysteine, hypotaurine and catalase in this regard (Baker *et al*., 1996). Glutathione and hypotaurine have also been shown to protect human spermatozoa from hydrogen peroxide mediated stress by Donnelly *et al*. (2000). However, if this strategy is pursued, great care must be taken in selecting the most appropriate antioxidants for clinical use. For example, we have previously shown that the major antioxidant in green tea (epigallocatechin gallate) can covalently cross-link sperm DNA to the point where fertilization would be impossible (Bennetts *et al*., 2008). High doses of the same antioxidant have also been shown to inhibit IVF in a porcine model (Spinaci *et al*., 2008). Furthermore, since ROS play an important role in regulating the signal transduction cascades that drive sperm capacitation, we should ensure that any antioxidants employed *in vitro* do not compromise the fertilizing potential of these cells (De Lamirande and Gagnon, 1993; Aitken *et al*., 1995a, b).

Combating oxidative stress *in vivo*

If oxidative stress is such a prominent feature of DNA damage in human spermatozoa then surely antioxidant administration should be part of the cure. Recent analyses of DNA damage in spermatozoa following exposure to various antioxidant preparations *in vivo* have provided some support for this concept (Greco *et al*., 2005; Tremellen, 2008). Additional studies are now required involving the careful selection of patients exhibiting high levels of oxidative DNA damage in their germ line, using robust recruitment criteria such as the cellular expression of 8OHdG. The fact that Greco *et al*. (2005) recorded beneficial results with an antioxidant regimen when the only selection criterion employed was high levels of DNA damage in the patients'

spermatozoa, adds yet more weight to the notion that a major cause of DNA damage in human spermatozoa is oxidative stress.

Summary and future directions

DNA damage in human spermatozoa is associated with a range of adverse clinical consequences including infertility, miscarriage and morbidity in the offspring. The origins of this damage are not clearly understood but in light of recent findings we have advanced a two-step hypothesis for its possible cause. The first step involves a defect in spermiogenesis as a consequence of which cells are prematurely released from the germinal epithelium in a dysfunctional state. These 'dysmature' cells are distinguished by excess residual cytoplasm, significant nucleohistone presence in the chromatin, aberrant protamination, high levels of unsaturated fatty acids, and poor zona binding potential. Defective chromatin remodelling renders these cells particularly susceptible to oxidative attack and the induction of DNA damage. Moreover, these cells are proposed to have a tendency to default to a programmed pro-senescence pathway resembling apoptosis, which involves the elevated generation of ROS by the sperm mitochondria. Of course this model may not apply in all cases of DNA damage and other mechanisms may also contribute to the overall pathological picture. Nevertheless, the scheme set out in Fig. 3 provides a theoretical framework with which to further investigate this important pathological process.

Future studies could address the fundamental molecular basis of sperm chromatin remodelling so that insights might be gained into the mechanisms responsible for the aberrant spermiogenesis seen in infertile males. Furthermore, since poor protamination (a spermiogenesis defect) and the retention of excess residual cytoplasm (a spermiation defect) are commonly encountered in defective human spermatozoa, the relationship between these two processes needs to be clarified, including the triggers responsible for timing the release of spermatozoa from the germinal epithelium. The triggers for mitochondrial ROS generation also need to be determined and the relationship between this process and the induction of 'apoptosis', carefully investigated. The role of other sources of ROS generation such as NOX 5 in the creation of oxidative stress in the germ line also need to be critically investigated (Bánfi *et al*., 2001; Baker and Aitken, 2004; De Iuliis *et al*., 2006). Finally, important questions are raised by the heterogeneous nature of human spermatozoa, which need to be explored in more depth (Muratori *et al*., 2008). Thus there is still uncertainty as to whether indices of sperm quality such as morphology or DNA damage are of diagnostic value because they tell us about the quality of an individual gamete or because they reflect the underlying quality of the spermatogenic process. This issue has particular relevance to the practice of ICSI, where emphasis is placed on selecting spermatozoa that appear to be normal. It may be significant that Sperm Chromatin Structure Assay values are of diagnostic significance when measured in unfractionated semen, but of no diagnostic value when performed on the washed selected cells used for insemination (Bungum *et al*., 2008). Such data clearly suggest that measures of sperm DNA damage are telling us as much about the quality of the underlying spermatogenic process as the fertilizing potential of individual spermatozoa (Bungum *et al*., 2008; Zini and Sigman, 2009).

From a clinical perspective, we simply cannot ignore animal data that provide an incontrovertible link between DNA damage in spermatozoa and defects in embryonic development. As a consequence, avenues should be urgently pursued for the remediation of this damage in a clinical context, as a matter of 'best practice'. Specifically, there is now an urgent need for double-blind, randomized, crossover trials of the efficacy of antioxidant treatment in reducing DNA damage in the spermatozoa of infertile males. Moreover, it is absolutely critical that such studies are conducted on patients for whom there is good evidence of oxidative stress in their germ line including measures of oxidative DNA base adduct formation (8OHdG) and lipid peroxidation (malondialdehyde, 4-hydroxyalkenals or 15-F(2t) isoprostane).

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