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STANDPOINT REVIEW

Not every sperm is sacred; a perspective on male infertility

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ABSTRACT: This article is a personal perspective on male infertility, a condition that is not only extremely prevalent but also a major reason for couples to resort to ART. The introduction of ICSI as a form of facilitated fertilization had a revolutionary impact on our capacity to treat cases of male infertility associated with severely compromised semen quality. However, the widespread use of this technique is also thought to pose risks in terms of the incidence of miscarriage, the health and wellbeing of the offspring and perpetuation of the infertile phenotype into future generations. Furthermore, the advent of ICSI curtailed intellectual interest in the underlying aetiology of male infertility or the development of non-invasive therapeutic strategies that target the male patient rather than the physical deployment of his gametes. As a consequence, progress on elucidating the pathological mechanisms responsible for male infertility has been extremely slow. Genetic and/or epigenetic defects are certainly involved in many cases and may involve mutations/splicing defects affecting the integrity of the testicular RNA profile, as well as the overall kinetics of the transcription process. In addition, spermatogenesis is disrupted by a variety of factors (age, smoking, obesity) many of which are thought to influence fertility and the integrity of sperm DNA through the creation of oxidative stress. Determining the relative contributions of oxidative stress and genetic/epigenetic mutations to the aetiology of male infertility will be a major focus for future research in this important but neglected area

Key words: human spermatozoa / infertility / hamster oocyte penetration test / ART / ICSI / oxidative stress / lipid peroxidation / oxidative DNA damage / gene mutation / Y-chromosome deletion

In the beginning

I never intended to be an andrologist. Both my PhD and postdoctoral work had been on the uterine control of preimplantation development and I had fully intended to continue my work on endometrial biochemistry when I joined Roger Short's MRC Reproductive Biology Unit in Edinburgh in 1977. However, after several months waiting patiently in gynecology wards for some endometrial tissue to drop into my stainless-steel bowl, I finally admitted defeat and started to think about other areas of human reproduction, where the supply of clinical material might be less limiting. Following discussion with Roger, we decided on two priority areas of research—the development of novel approaches to contraception, particularly vaccines, and the investigation of male infertility.

At this time, 'the male factor' was recognized as a very common cause of human infertility responsible for a great deal of private suffering and pain but a complete mystery as far as the underlying aetiology was concerned. In 1977, the only diagnostic tool that we possessed was the conventional descriptive semen profile and the only weapon in our therapeutic armamentarium was artificial insemination by donor (AID) employing cryostored human semen. The MRC Unit had just recruited David Richardson, one of the pioneers of AID and the demand for such services was high. In 1977, there were more than 2000 referrals for AID and more than 200 babies born as a result of this procedure (Richardson, 1980). Despite the demand, there was very little formal support for such services at the time and the activities of AID centers were not closely regulated. AID was a temporary, almost clandestine, solution to a problem—but it was not a cure for the disease.

Prevailing wisdom on semen quality

What did we know about male infertility at the time? The truth is, very little. We were aware that male infertility was generally associated with defects in the conventional semen profile that could influence the motility of the spermatozoa, their number or their morphology. However, we had precious few insights into the origins of these changes or their true significance. The prevailing andrological wisdom in 1977 was that

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infertility was really just a question of sperm number—a patient either generated a sufficient number of morphologically normal, motile spermatozoa to establish a pregnancy, or he did not. This kind of binary thinking sits behind the threshold values for sperm number, morphology and motility which have been promulgated by the World Health Organization—and argued about to this very day (Zhang *et al.*, 2014). Such thinking also led to futile attempts to treat infertility by inseminating pooled successive ejaculates in order to compensate for the low sperm numbers found in oligozoospermic males. Even as late as 1990, papers were appearing extoling the virtues of combining of multiple ejaculates from oligozoospermic males to increase the median total number of motile sperm in preparation for AI (Tur-Kaspa *et al.*, 1990). Unfortunately, such well-intentioned strategies never met with significant success because the underlying principle was flawed (Speichinger and Mattox, 1976). Male fertility was never simply a question of sperm number.

This conclusion was brought home years later when we were conducting trials in Edinburgh on hormonal approaches to male contraception. In these studies, we were injecting exogenous testosterone enanthate to suppress gonadotrophin production and temporarily impair spermatogenesis. During the course of these trials, we were surprised to see pregnancies attributable to treated men with sperm counts that had been suppressed well into the pathological range (~3 × 10^6/ml) (Wallace *et al.*, 1992). Similar results have been observed by others (World Health Organization, 1996) and serve to remind us that fertility is poorly correlated with sperm concentration. If the spermatozoa are normal, then sperm counts well into the sub-normal range are perfectly capable of establishing a pregnancy. Exactly the same conclusion can be drawn from the pregnancies achievable with Kallmann syndrome patients following treatment with exogenous gonadotrophins, where pregnancies are observed despite pathologically low sperm counts (Dwyer *et al.*, 2015; Rohayem *et al.*, 2016). Another perfect illustration of the weakness inherent in the conventional semen profile can be found in a recent analysis of fertility in post-vasovasostomy patients. In this group of males, spontaneous pregnancies were reported in 15% of patients with a sperm concentration of <5 million/ml, 21.3% with a sperm motility of <10%, and 14.8% with a normal morphology of <1%, (Majzoub *et al.*, 2017). The authors correctly conclude that the thresholds of semen normality defined by the World Health Organization (2010) may not adequately reflect fertility in vasectomy–reversal patients in whom the underlying spermatogenic process is normal. All of these examples point to the same conclusion—*that you cannot determine the fertilizing potential of a sperm population using descriptive semen parameters such as count, motility or morphology*. Fundamentally, not all normal looking, motile spermatozoa are created equal.

Of course, this does not mean that the conventional semen profile has no value. On the contrary, when carefully performed it can clearly generate data on relative semen quality for a given population. Using the fifth centile of fertile men (time to pregnancy of ≤12 months) as the lower threshold of normality (Cooper *et al.*, 2010; Auger *et al.*, 2016), it is perfectly possible to determine whether a patient's semen quality is 'normal' in relation to males of proven fertility within the same population. Whether 'sub-normal' in this context is equivalent to 'subfertile' is more difficult to ascertain. As a prognostic tool the semen profile lacks precision because it can only ever provide an indirect indication of the relative efficiency of the underlying sperm production process. It is not so much the appearance of the spermatozoa that we should be focusing on, but rather, their capacity for fertilization.

Yanagimachi and the zona-free hamster oocyte penetration test

In 1976 the revered gamete biologist, Ryuzo Yanagimachi, published a sentinel paper on the fertilization of hamster ova by the spermatozoa of different species, including man (Yanagimachi *et al.*, 1976). This article was, literally, so incredible at the time, that it was initially rejected by at least one of the field's leading journals on the basis of being 'impossible'. However, further research demonstrated that the ability of human spermatozoa to fuse with zona-free hamster ova was not only possible but physiologically meaningful, in that the architecture of sperm–oocyte union appeared to replicate exactly the homologous situation, i.e. that the equatorial segment of capacitated acrosome-reacted spermatozoa suddenly acquires the ability to bind to, and fuse with, the vitelline membrane of an MII oocyte (Fig. 1). This bioassay gave us a powerful new tool to look at the fertilizing potential of spermatozoa and really test the hypothesis that not all spermatozoa are created equal. We developed standardized protocols for conducting the assay that were promulgated with the help of the World Health Organization (Aitken, 1986) and demonstrated that the assay was capable generating data of clinical relevance in long-term prospective trials and donor insemination programs (Irvine and Aitken, 1986; Aitken *et al.*, 1991). However, despite its biological relevance, this assay was never going to be a diagnostic test for routine clinical use; it was far too complex, labor intensive and difficult to standardize. Rather, the hamster oocyte penetration assay was an excellent

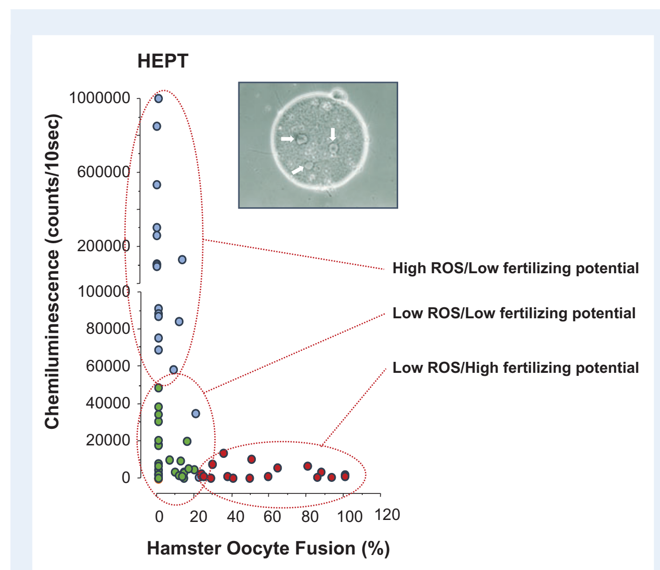

Figure 1 Relationship between the generation of ROS by human spermatozoa and their capacity for sperm–oocyte fusion as measured by the zona-free hamster oocyte penetration test. Insert shows a zona-free hamster oocyte possessing several decondensed human sperm heads in its cytoplasm (arrowed). Each data point is an independent patient who can be allocated to one of three general categories: (i) high ROS, low fertilizing potential (blue) where oxidative stress is implicated in the patients' infertility, (ii) low ROS, low fertilizing potential(green) where the infertility has some other cause and (iii) low ROS, high fertilizing potential (red) where the spermatozoa appear normal. After Aitken *et al.* (1989a).

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experimental research tool that allowed us to gain mechanistic insights into the nature of defective sperm function.

An important, but overlooked, feature of this assay was that the relationship between motile sperm concentration and fertilization rate could be accurately modeled using Poison distribution theory (Aitken and Elton, 1984). We used this facility to design experiments in which we could compare the fertilizing potential of motile spermatozoa from normal fertile donors with motile spermatozoa collected from patients exhibiting idiopathic asthenozoospermia (<40% motility), asthenozoospermia associated with varicocele and oligoaesthenozoospermia (<20 $\times 10^6$ spermatozoa/ml and <40% motility). On a motile-sperm-to-motile sperm comparative basis, the Poison distribution model conclusively demonstrated that not all motile spermatozoa are equivalent; those recovered from fertile males possessed significantly more fertilizing potential than those recovered from infertile subjects (Aitken and Elton, 1986).

So, if all motile spermatozoa are not created equal, what is it that defines the difference in functional competence? A key insight into this conundrum came when we discovered that the difference in oocyte-fusion rates between normal and infertile males, was still evident when calcium entry was artificially induced using the divalent cation ionophore, A23187 (Aitken *et al.*, 1984, 1993a). Applying the Poisson model, we could clearly show that the response of motile spermatozoa from infertile males to calcium ionophore exposure was significantly compromised relative to fertile donors (Aitken *et al.*, 1984). Such results sharply focused attention on damage to the sperm plasma membrane that might impair their capacity to respond to a calcium influx with an increase in fusogenicity. This line of thinking ultimately led us into the complex landscape of reactive oxygen species (ROS) generation and oxidative stress.

A free radical theory of male infertility

As early as 1979, Thaddeus Mann published a landmark paper with Roy Jones and Dick Sherins highlighting the vulnerability of human spermatozoa to oxidative stress (Jones *et al.*, 1979). The significance of this work became apparent when we discovered that the generation of ROS was significantly elevated in sperm suspensions from infertile males (Aitken and Clarkson, 1987). The excessive generation of ROS by these cells was found to induce peroxidative damage in the sperm plasma membrane as a consequence of which the spermatozoa lost their capacity to respond to calcium signals with an increase in sperm–oocyte fusion. Thus experimentally, we could show that if normal spermatozoa were artificially exposed to ROS *in vitro*, they lost their ability to fertilize oocytes following stimulation with A23187, precisely recapitulating the *in vivo* situation (Aitken *et al.*, 1993b). Similarly, the induction of lipid peroxidation in normal spermatozoa following exposure to catalytic quantities of Fe (II), resulted in the production of populations of spermatozoa that were incapable of responding to A23187, just like the infertile population (Aitken *et al.*, 1993c). The fundamental conclusion of these studies was that the loss of sperm functionality in cases of male infertility involved some element of oxidative stress. Figure 1 demonstrates the strength of this association, with most defective sperm–oocyte fusion associated with high levels of ROS generation. Importantly, this phenomenon did not account for every case of defective sperm function in the cohort of patients examined—but it did account for around 55% of cases where the fertilizing capacity of the spermatozoa was profoundly impaired (Aitken *et al.*, 1989a,b).

Measuring ROS generation

The major caveat with this type of analysis was that it involved the use of luminol-dependent chemiluminescence to detect the cellular generation of ROS. The luminol technique is highly sensitive but suffers from two major problems. Firstly, it is not specific for any particular species of ROS and secondly it will generate very strong signals in the presence of leukocytes, which contaminate every human sperm suspension. In a very long, painstaking piece of work, we carefully dissected out the contributions of leukocytes and spermatozoa to the chemiluminescence signal and concluded that while leukocyte contamination is a major contributor to overall ROS generation by human sperm suspensions (Kessopoulou *et al.*, 1992), it is not the only source (Aitken and West, 1990; Aitken *et al.*, 1992). This conclusion was also subsequently reached by others (Whittington and Ford, 1999) and was put beyond doubt by the advent of flow cytometry procedures that allowed specific focus on ROS generation by the sperm cell population (De Iuliis *et al.*, 2006; Purdey *et al.*, 2015).

A variety of well-authenticated fluorescent probes are now available for detecting ROS generation by human sperm populations. One of the first to be evaluated was dihydroethidium, the 2-electron reduction product of ethidium (De Iuliis *et al.*, 2006). In this study high-performance liquid chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy, and spectrofluorometry were all used to demonstrate that human spermatozoa generate the superoxide-specific product, 2-hydroxyethidium, from DHE. While this study provided definitive evidence for superoxide generation by human spermatozoa there are two reservations with the use of this probe in clinical practice. The first is that the probe has to be used in conjunction with a vitality stain such as SYTOX green. The reason for this is that commercial preparations of DHE contain trace amounts of the parent ethidium compound, which will stain the nuclei of dead cells, red, irrespective of whether or not they are generating ROS. In practice, this assay only has biological meaning when focused on the subpopulation of viable spermatozoa. Secondly, we should acknowledge that a wide variety of oxidation events will convert DHE into the DNA-sensitive fluorochrome, ethidium, not just superoxide anion. Since flow cytometry will not distinguish between signals generated by 2-hydroxyethidium (superoxide specific) and ethidium (non-specific oxidation) care should be take when ascribing the results to ROS generation. These caveats (the need to focus on live cells and awareness of the possibility of non-specific oxidation) apply to most of the probes currently used to detect ROS by flow cytometry.

Sources of ROS in mammalian spermatozoa

The sperm mitochondria as ROS generators

The source of ROS in defective spermatozoa is also a matter for conjecture although there seems to be general agreement that the leakage of electrons from the mitochondrial electron transport chain (ETC) is a significant factor (Koppers *et al.*, 2008; Cassina *et al.*, 2015). Such spontaneous mitochondrial ROS generation is known to lead to a loss

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of mitochondrial membrane potential, the induction of lipid peroxidation and the suppression of sperm movement (Chai et al., 2017). In this context, at least three major pathways are known to be involved in eliciting mitochondrial ROS generation by spermatozoa:

- *Exposure to amphiphiles that disrupt mitochondrial electron transport*. Free unesterified polyunsaturated fatty acids (PUFA) induce high levels of mitochondrial ROS generation, when added to purified populations of human spermatozoa. This relationship is biologically and clinically important because powerful correlations have been observed between the spontaneous generation of ROS by human spermatozoa and their free PUFA content (Koppers et al., 2008). A key structural determinant of the PUFA that are capable of enhancing mitochondrial ROS generation is that they should possess amphipathic properties, i.e. possess a hydrophilic region that will locate to the surface of the membrane and a hydrophobic domain that will penetrate into the membrane's interior (Aitken et al., 2006). The embedding of free PUFA in the inner mitochondrial membrane appears to perturb electron flow along the ETC, leading to electron leakage and superoxide anion generation. Fatty acids are not alone in this context; we have also discovered a variety of natural (retinoids) and unnatural (parabens) amphipathic compounds that will trigger mitochondrial ROS via the same mechanism.
- *Chemical adduction of mitochondrial proteins with powerful electrophiles*. Examples of compounds that form adducts with mitochondrial proteins are 4-hydroxynonenal (4HNE) or acrolein, both of which are aldehydes generated as a consequence of lipid peroxidation (Aitken et al., 2012). Such activity explains a very important attribute of oxidative stress in spermatozoa—it is a self-perpetuating phenomenon. Once oxidative stress is initiated by such factors as antioxidant depletion, exposure to free radical-generating leukocytes or treatment with compounds such as PUFA, the stressed state becomes perpetuated because the lipid aldehydes generated as a consequence of the peroxidation process trigger yet more ROS generation from the mitochondria following the formation of adducts with proteins within the ETC such as succinate dehydrogenase (Fig. 2; Aitken et al., 2012). ROS–induced-ROS-generation has been observed by many authors (du Plessis et al., 2010) and is explained by this mechanism. Furthermore, this phenomenon would also explain leukocyte-induced ROS generation by human spermatozoa (Saleh et al., 2002).

  Importantly lipid aldehydes are not the only electrophiles capable of generating this activity. Gossypol, a constituent of cotton seed oil once proposed as a male contraceptive, will also activate ROS generation by alkylating key proteins within the ETC (Aitken et al., 2016b). The homocysteine cyclic congener, homocysteine thiolactone is another compound capable of activating mitochondrial ROS generation as a consequence of its ability to bind proteins in the ETC (Aitken et al., 2016a). The negative impact of homocysteine is exacerbated by oxidative stress because paraoxonase 1 (PON-1), the major enzyme responsible for removing thiolactone from proteins, is another target for alkylation by lipid aldehydes generated as a consequence of oxidative stress. Exposure to 4HNE therefore leads to homocysteine accumulation in spermatozoa and the further stimulation of mitochondrial ROS generation. There is an embryonic literature on the relationship between hyperhomocysteinaemia and male infertility (Ebisch et al., 2006; Ge et al., 2008) that might be worthy of exploration in future studies.
- *Reduced mitochondrial expression of the prohibitin complex*. The latter is a macromolecular structure within the inner mitochondrial membrane that is thought to possess a scaffold-like function, maintaining the structural integrity of complexes involved in the ETC. Reduction of prohibitin levels is seen in the spermatozoa of infertile patients in a manner that is negatively correlated with the induction of mitochondrial ROS generation (Chai et al., 2017). Since prohibitin is vulnerable to oxidative attack (Opii et al., 2007) and can be down-regulated in the testes by oxidative stress (Li et al., 2016), this may be yet another example of a ROS-mediated attack on the male germ line leading to changes that perpetuate the stressed state.
- *Opening of the mitochondrial permeability transition pore* (Treulen et al., 2015). Opening of the pore leads to a loss of mitochondrial membrane potential, the dysregulation of electron flow through the ETC and the consequential generation of ROS. Once again, oxidative stress can precipitate opening of the inner mitochondrial membrane pore, via mechanisms that can be reversed by the presence of melatonin (Waseem et al., 2016).
- *The induction of apoptosis*. One part of the puzzle that we should not lose sight of is that mitochondrial ROS generation is a key feature of the truncated apoptotic pathway engaged by spermatozoa when they become stressed. There are no known chemical triggers for apoptosis in spermatozoa that have physiological relevance; rather, apoptosis is their default state. Spermatozoa will automatically engage this pathway unless they are prevented from doing so by the action of prosurvival factors. The key here seems to be the phosphorylation status of AKT1 (Fig. 3). As long as the latter is phosphorylated, the cells are alive and functional and downstream effectors of apoptosis such as Bcl-2-associated death promoter (BAD) are silenced. The key to maintaining AKT1 in a phosphorylated state is to ensure that the enzyme responsible for its phosphorylation, phosphatidylinositol 3-kinase (PI3 kinase), is always in an activated state. There are probably many prosurvival factors capable of performing this role; prolactin and insulin (Aquila et al., 2005; Pujianto et al., 2010) are the ones we know about, but there are undoubtedly many others. Conversely, if PI3 kinase activity is suppressed with a compound such as wortmannin, then AKT1 becomes rapidly dephosphorylated and the cells enter the intrinsic apoptotic pathway characterized by caspase activation in the cytosol, annexin V binding to the cell surface, mitochondrial ROS generation, cytoplasmic vacuolization, oxidative DNA damage and motility loss (Koppers et al., 2011).

NADPH oxidase

Apart from the sperm mitochondria there are a variety of other potential sources of ROS in spermatozoa that are of considerable interest but unknown significance. For many years, the notion of an NADPH oxidase in spermatozoa has been promoted as a possible source of ROS. There is no doubt that spermatozoa do contain NADPH oxidases including NOX5, a calcium regulated NADPH oxidase which is expressed at high levels in both the testes and spleen (Bánfi et al., 2001). In keeping with the presence of such a calcium-dependent oxidase exposure of suspensions of human spermatozoa to calcium ionophores such as A23187 or ionomycin, has been reported to trigger ROS production (Aitken and Clarkson, 1987; Musset et al., 2012), However, it should be acknowledged that these studies did not involve the use of purified sperm preparations from which all traces of leukocyte contamination had been removed. It is therefore possible that the putative NADPH oxidase detected in these sperm suspensions was, in fact, due to leukocyte contamination. The inhibitory action of apocynin (a NOX inhibitor) on ROS generation by human spermatozoa suffers from the same problem because the chemiluminescent signal recorded in these experiments might have been generated by contaminating leukocytes (Donà et al., 2011). The fact that diphenylene iodonium

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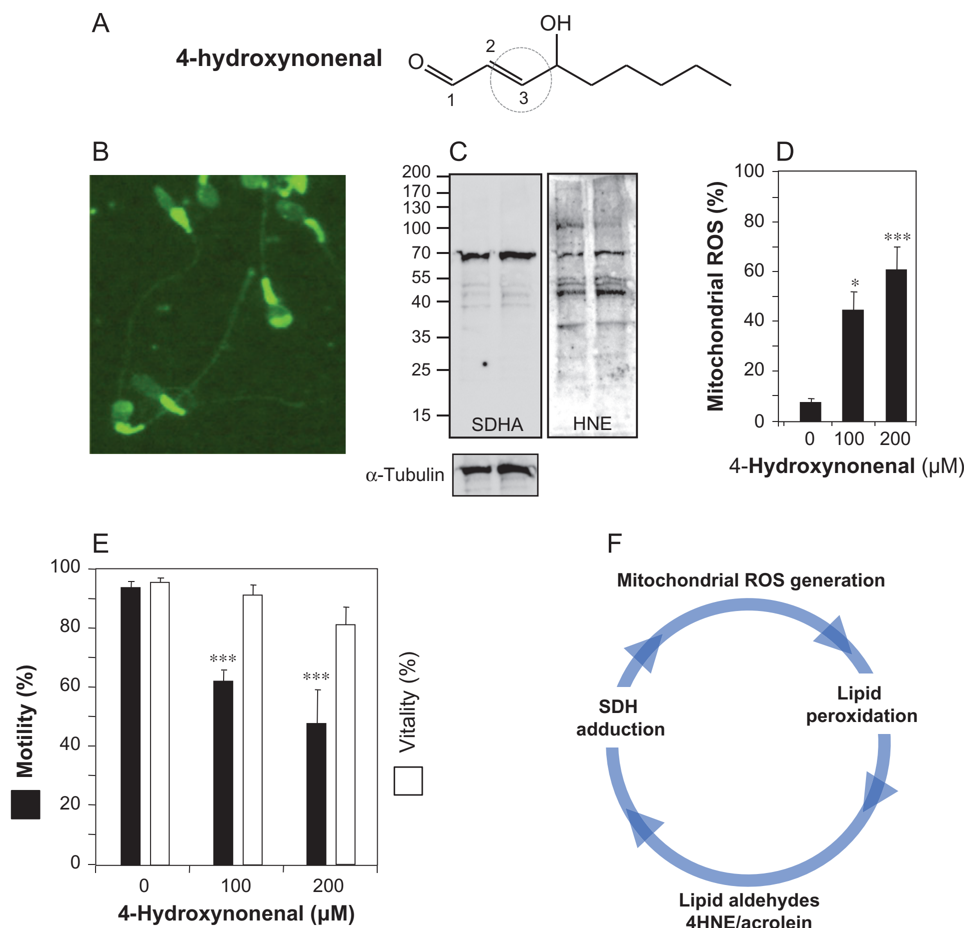

Figure 2 Oxidative stress in human spermatozoa is a self-perpetuating process mediated by the aldehyde products of lipid peroxidation such as 4HNE. (A) 4HNE possesses an electrophilic carbon atom at position 3 (circled) that will bind to a variety of nucleophilic targets including the histidine, lysine and cysteine residues of proteins. (B) The targets for electrophilic adduction are widely distributed around the cell but particularly prevalent in the acrosome and midpiece. (C) Western blot analysis reveals several major targets, one of which is succinic acid dehydrogenase (SDHA). (D) the biochemical consequence of such adduction is the spillage of electrons from the mitochondrial electron transport chain leading to a dose-dependent increase in ROS generation. (E) The oxidative stress generated in this manner, along with the direct impact of protein adduction on the functionality of key axonemal proteins such as dynein heavy chain (Baker *et al.* 2015), results in a significant loss of sperm motility. (F) The generation of mitochondrial ROS as a consequence of mitochondrial protein adduction ensures that oxidative stress is a self-perpetuating state in these cells.

suppresses ROS generation by human spermatozoa is also not definitive evidence for an NADPH oxidase because this flavoprotein inhibitor can also suppress ROS generation by mitochondria (Li and Trush, 1998). Furthermore, the use of exogenous NADPH to stimulate lucigenin chemiluminescence by human spermatozoa (Aitken *et al.*, 1997) is not evidence of an oxidase, because the signals generated under these conditions reflect the direct reduction of the probe by oxidoreductases; cytochrome b5 reductase in the case of NADH and cytochrome p450 reductase in the case of NADPH (Baker *et al.*, 2004, 2005) Moreover, superoxide anion generation has not be detected following the addition of exogenous NADPH to human spermatozoa (de Lamirande *et al.*, 1998).

Amino acid oxidase and lipoxygenase

Additional potential sources of ROS in spermatozoa include amino acid oxidases and lipoxygenase. L-amino acid oxidases were, in fact, the first enzymes to be associated with ROS generation in spermatozoa as a result of the pioneering work of Tosic and Walton in the 1940s and 50s (Tosic and Walton, 1950). These authors demonstrated that the loss of motility observed in bovine spermatozoa in the presence of egg yolk extenders could be attributed to hydrogen peroxide generation by an amino acid oxidase exhibiting a particular affinity for aromatic amino acids such as phenylalanine. Tosic and Walton also found that the responsiveness of this enzyme system was enhanced by cell death, which presumably provided the oxidase with unfettered access to its substrate. We have found exactly the same enzyme activity in equine spermatozoa (Aitken *et al.*, 2015) and again demonstrated that dead spermatozoa are particularly responsive to the presence of substrates such as phenylalanine and that the ROS generated under such conditions can compromise the functionality of live cells in the same suspension—but what of human spermatozoa? We have detected this enzyme activity in human sperm cells (Houston *et al.*, 2015) and found that unlike equine and bovine

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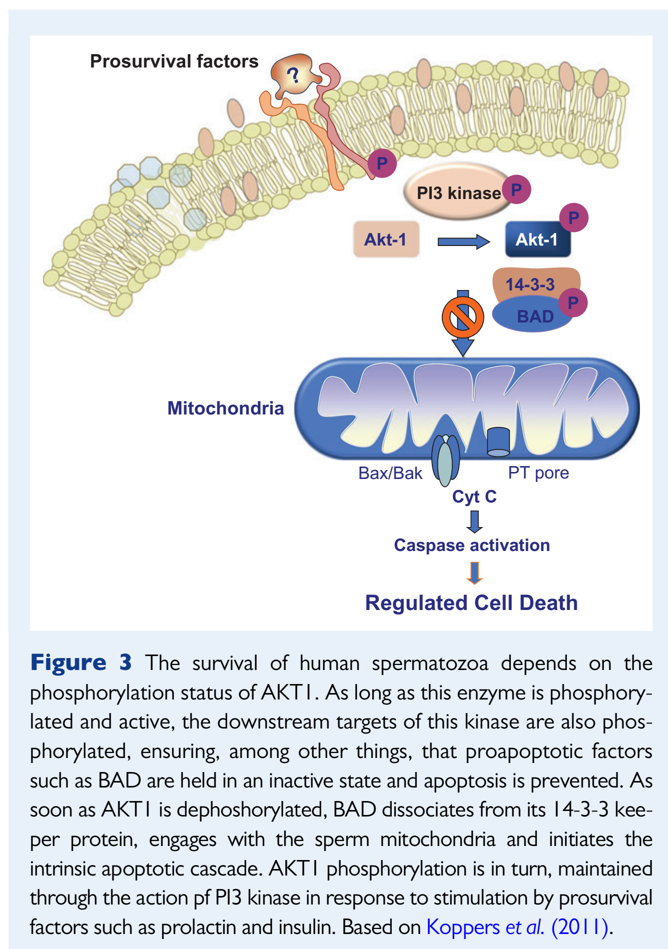

Figure 3 The survival of human spermatozoa depends on the phosphorylation status of AKT1. As long as this enzyme is phosphorylated and active, the downstream targets of this kinase are also phosphorylated, ensuring, among other things, that proapoptotic factors such as BAD are held in an inactive state and apoptosis is prevented. As soon as AKT1 is dephoshorylated, BAD dissociates from its 14-3-3 keeper protein, engages with the sperm mitochondria and initiates the intrinsic apoptotic cascade. AKT1 phosphorylation is in turn, maintained through the action pf PI3 kinase in response to stimulation by prosurvival factors such as prolactin and insulin. Based on Koppers *et al.* (2011).

spermatozoa, this enzyme is rapidly lost from non-viable cells. However, stimulation of intracellular hydrogen peroxide generation with extracellular phenylalanine was found to enhance capacitation and the ability of the spermatozoa to acrosome react suggesting a physiological role for the IL4I1 l-amino acid oxidase in human spermatozoa, potentially driven by the free amino acids present in the secretions of the female reproductive tract (Houston *et al.*, 2015). There is no evidence to suggest that IL4I1 is in any way involved in the ROS generation seen in the spermatozoa of subfertile males.

Another possible trigger for ROS generation in human spermatozoa is lipoxygenase. This enzyme (ALOX15) is known to generate ROS in several cellular systems and its inhibition in male germ cells leads to a significant reduction in both mitochondrial and cytoplasmic ROS generation, as well as a dramatic reduction in 4HNE (Bromfield *et al.*, 2017). This observation is in keeping with the increased availability of free fatty acids in the spermatozoa of subfertile males and the fact that exposure of spermatozoa to free unesterified PUFA, such as arachidonic acid, trigger the rapid generation of ROS by human spermatozoa (Koppers *et al.*, 2010). This is an important area for further study since ALOX15 might be a potential target for ameliorating oxidative stress in defective human spermatozoa. The clear link between the retention of excess residual cytoplasm by human spermatozoa and subsequent ROS generation (Gomez *et al.*, 1996) provides a potential mechanism by which defective spermatogenesis might generate cells that are over-endowed with enzymes such as ALOX15 that might then trigger increased ROS generation and oxidative stress.

Importance of peroxynitrite

Clearly there is no simple answer when it comes to addressing the source of ROS in human spermatozoa and we should keep an open mind as to which sources dominate at different stages of germ cell development. Moreover, while this review has focused on ROS it should be acknowledged that, *in vivo*, reactive nitrogen species (RNS) are also important (Uribe *et al.*, 2015). Indeed, peroxynitrite is a very powerful oxidant generated by the reaction of superoxide anion with nitric oxide that has been postulated to mediate a continuous capacitation-apoptosis highway (Fig. 3). According to this model, peroxynitrite generated in spermatozoa following ejaculation induces several of the early features of capacitation including cholesterol efflux from the plasma membrane, PS exposure, presentation of zona-receptor complexes on the sperm surface, inactivation of tyrosine phosphatases, and stimulation of cAMP production (Aitken, 2011). However, if spermatozoa capacitated via this mechanism fail to meet an egg, then the continued generation of peroxynitrite will carry the spermatozoa over the top of the hill of functional competence and down into the cul de sac of apoptosis. This is the senescent fate that awaits a vast majority of spermatozoa in the ejaculate.

A loss of fertilizing potential is, of course, not the only impediment created by oxidative stress in the male germ line. Of even greater significance in some ways, is the induction of genetic and epigenetic mutations that influence the development normality of the offspring.

DNA damage in human spermatozoa

The importance of oxidative stress

The physical architecture of spermatozoa prevents endonucleases released from the mitochondria (e.g. endonuclease G [Endo G] or apoptosis inducing factor [AIF]) or activated in the cytosol (e.g. caspase-activated DNase [CAD]) during the intrinsic apoptotic cascade from penetrating the sperm nucleus (Fig. 4). This is because spermatozoa are structurally unique in that the mitochondria and nucleus are in different compartments of the cell. As a result of this unique structural feature, the sperm nucleus is largely shielded from the powerful nucleases generated in the mitochondria and cytoplasm during apoptosis. Charged ROS such as the superoxide anion are also prevented from penetrating the sperm nucleus; however, this limitation will not apply to uncharged ROS, such as hydrogen peroxide (Fig. 4).

These considerations have major implications for the aetiology of DNA damage in spermatozoa because DNA can only be fragmented by two mechanisms: (i) exposure to ROS or (ii) the action of nucleases. If nucleases are physically trapped in the midpiece of the cell then the only mechanism by which DNA damage can be induced is by oxidative attack. It is for this reason that we have asserted that most, if not all, DNA damage in spermatozoa is instigated by ROS and reflected by the formation of oxidative DNA adducts, particularly 8-hydroxy,2′deoxyguanosine (8OHdG) (Kodama *et al.*, 1997; Aitken *et al.*, 2010). In contrast, mitochondrial DNA is not protected from nucleases released during apoptosis. This feature, plus the fact that mitochondrial DNA is not protected by complexation with proteins, make this particular form of DNA an excellent marker for genetic damage in spermatozoa (Sawyer *et al.*, 2003), which is currently under-exploited.

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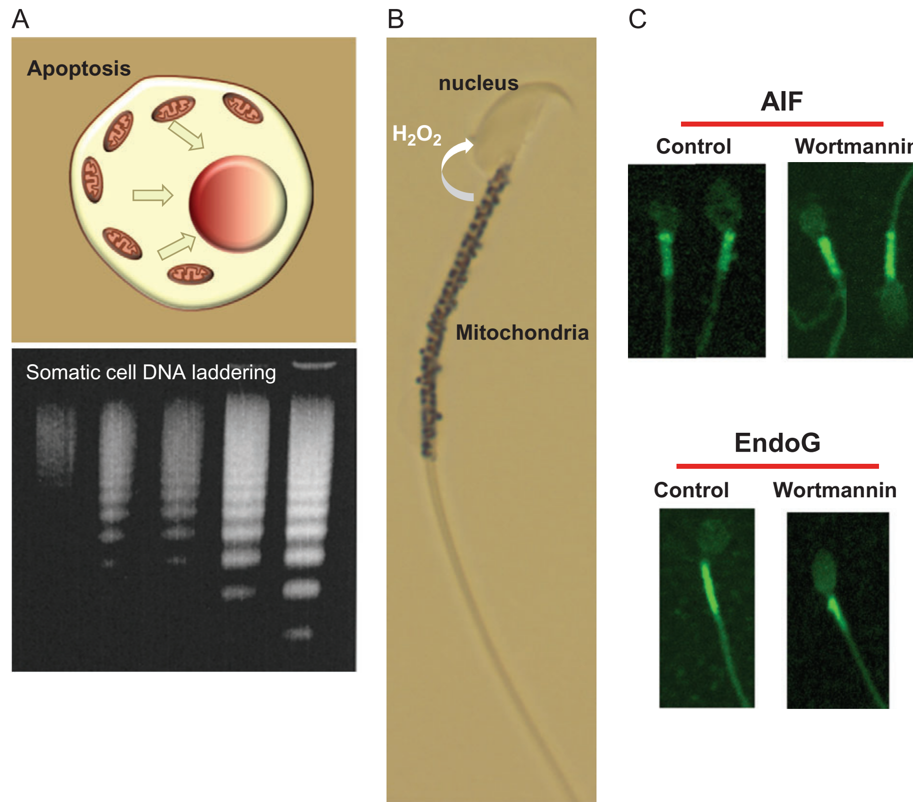

Figure 4 The unusual architecture of spermatozoa influences the induction of DNA damage in these cells. (A) In somatic cells, endonucleases released from the mitochondria or activated in the cytosol during apoptosis, move into the nuclear compartment and cut the DNA, generating the characteristic laddering pattern following electrophoresis. (B) In spermatozoa, the mitochondria (labeled black) and most of the cytoplasm are located in the midpiece of the cell, away from the nuclear compartment. (C) As a result of this unique arrangement, nucleases associated with apoptosis such as Apoptosis Inducing Factor (AIF) and Endonuclease G (EndoG) remain resolutely locked in the midpiece of the cell when apoptosis is induced by wortmannin. Only a membrane permeant molecule such as hydrogen peroxide (B) has the capacity to move from the sperm midpiece to the nucleus during apoptosis. It is for this reason that the instigation of DNA damage in spermatozoa is usually oxidative.

When DNA damage does occur, it may have important implications for the offspring because spermatozoa with damaged DNA can still be capable of fertilization (Aitken *et al.*, 1998, 2013). In this context it is worth reflecting that sperm chromatin is not uniformly vulnerable to oxidative attack. There is a distinct variation between chromosomes in their susceptibility to oxidative base damage (8OHdG density) driven by the highly ordered and specific organization of sperm chromatin. Most sperm domains that are vulnerable to oxidation lie at the periphery of the sperm nucleus, where the telomeres are highly concentrated, or close to the sperm midpiece, in the vicinity of free radical-generating mitochondria. Furthermore there are particularly vulnerable areas of the sperm nucleus, between the highly compacted protamine-enriched toroids, where compaction is reduced and the DNA is susceptible to oxidative attack because it is either naked or in a loosely compacted nucleosomal arrangement. These inter-toroid regions are particularly rich in short interspersed repeat elements (SINEs) and long interspersed repeat elements (LINEs) as well as centromeres and telomeres. In both mouse and human spermatozoa the sex chromosomes appear to be particularly well-protected from oxidative attack, while certain autosomes, by virtue of their composition and positioning within the sperm nucleus, are highly vulnerable. In the mouse chromosome 19 appears to be particularly vulnerable while in human spermatozoa the vulnerable chromosomal domain is 15q13–15q14, potentially encompassing the paternally inherited imprinting center that is 5′ to this region (Noblanc *et al.*, 2013; Kocer *et al.*, 2015; Xavier, M., unpublished observations). Importantly locus 15q13–15q14 maps to a chromosomal hotspot associated with variable cognitive and other neuropsychiatric expression, including conditions that we know are paternally driven and thus associated with defects in spermatozoa (e.g. epilepsy, autism spectrum disorder and spontaneous schizophrenia; Lowther *et al.*, 2017).

DNA repair in spermatozoa

Since the limited cytoplasmic space available in these highly specialized cells places constraints on the availability of antioxidant enzymes, one of the major defensive strategies employed by spermatozoa is to tightly complex the DNA with protamines. The latter serve as sacrificial antioxidants and also bind transition metals such as copper that would otherwise catalyze the formation of hydroxyl radicals and the induction of DNA fragmentation (Kasprzak, 2002; Aitken *et al.*, 2014). In keeping with this role for protamines, we have found that DNA

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fragmentation in human spermatozoa is inversely correlated with the level of DNA protamination as determined by the intercalating DNA dye, chromomycin A3 (De Iuliis *et al.*, 2009). The protective role of chromatin protamination has been repeatedly observed by others (Manochantr *et al.*, 2012; Fortes *et al.*, 2014; Ghasemzadeh *et al.*, 2015) resulting in the articulation of a 2-step hypothesis of DNA fragmentation in spermatozoa comprising: Step 1, where a defect in spermiogenesis leads to poorly protaminated chromatin, creating a state of vulnerability in the cell and then Step 2, during which this vulnerable chromatin becomes attacked by ROS, generating high levels of 8OHdG formation (De Iuliis *et al.*, 2009).

The 8OHdG formed during this process is targeted and removed by the first enzyme in the base excision pathway (BER) 8-oxoguanine DNA glycosylase (OGG-1). This glycosylase is associated with the sperm nucleus and mitochondria and actively removes 8OHdG, releasing this base adduct into the extracellular space (Smith *et al.*, 2013a,b). Remarkably, spermatozoa do not possess the downstream components of the base excision repair pathway, apurinic endonuclease 1 (APE1) and X-ray-repair-complementing-defective-repair-in-Chinese-hamster-cells 1 (XRCC1). The result of this truncated BER is therefore to generate an unresolved abasic site within the DNA duplex which cannot be prepared for the insertion of a new base by the spermatozoon; this task falls to the oocyte, which is richly endowed with both APE1 and XRCC1 (Smith *et al.*, 2013b). The presence of such unresolved abasic sites ultimately destabilizes the DNA and renders it vulnerable to DNA strand breakage.

One of the interesting practical consequences of this truncated BER pathway is that Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays cannot theoretically be applied to spermatozoa because this assay depends on terminal deoxynucleotidyl transferase (TdT) sequentially adding tagged nucleotides to the 3′-hydroxyl termini of DNA double strand breaks. In the absence of APE1 no 3′-hydroxyl groups will be available for the terminal transferase to target. In keeping with this assertion, when DNA damage is induced in human spermatozoa with hydrogen peroxide, the cells rapidly become 8OHdG positive and show signs of DNA damage with Sperm Chromatin Structure Assay (SCSA) but are negative with regard to the TUNEL assay. Despite this, there is abundant evidence that human spermatozoa *do* become TUNEL positive if they are defective (Mitchell *et al.*, 2011). This conundrum was resolved when it was found that spermatozoa exposed to an oxidative stress ultimately become TUNEL positive but it takes around 48 h for this change to manifest itself (Smith *et al.*, 2013b). Such observations are strongly supportive of the perimortem activation of a nuclease in the sperm nucleus, as suggested by others (Boaz *et al.*, 2008). However, the identity of this proposed intra-nuclear nuclease has yet to be determined.

Impact of sperm DNA damage on the next generation

One consequence of the truncated BER pathway in mammalian spermatozoa is that spermatozoa which are perfectly capable of fertilization will, nevertheless, carry both abasic sites and non-excised 8OHdG adducts into the egg at the moment of fertilization (Aitken *et al.*, 1998). The presence of such oxidized bases is a source of concern because they are extremely mutagenic, creating mutations in the offspring, typically involving G:C–A:T transversions, as a result of Hoogsteen base pairing. For example, the increase in childhood cancers seen in the offspring of male smokers (Lee *et al.*, 2009) may well reflect the elevated levels of 8OHdG seen in the spermatozoa of such subjects (Fraga *et al.*, 1996). Similarly, the increase in brain disorders such as autism, spontaneous schizophrenia and bipolar disease seen with paternal age, could also be associated with the increased levels of oxidative DNA damage seen in the spermatozoa as a consequence of the ageing process (Smith *et al.*, 2013a). Such relationships may also underpin the increased incidence of autism seen in children conceived by ICSI (Kissin *et al.*, 2015) reflecting the high levels of 8OHdG expression seen in the spermatozoa of male infertility patients (Meseguer *et al.*, 2008; Aitken *et al.*, 2010). These causative relationships between 8OHdG formation in spermatozoa, defective repair of this lesion in the oocyte and increased mutation frequencies in the offspring, is a critical component of the paternal contribution to abnormal development (Aitken *et al.*, 2004). It is at the heart of the impact of paternal ageing on the health and wellbeing of children and may also underpin the means by which a complex array of environmental, lifestyle and clinical factors impact on the developmental normality of children. Examples of external factors that could potentially influence offspring health by creating oxidative DNA damage in the male germ line include electromagnetic radiation (Houston *et al.*, 2016), obesity (Lane *et al.*, 2014), smoking (Fraga *et al.*, 1996) and a wide range of environmental pollutants including products from the chemicals industry (phthalate esters, bisphenol A) preservatives (parabens), insecticides (Firpronil) herbicides (alachlor) and therapeutic agents (paracetamol) (Grizard *et al.*, 2007; Khan *et al.*, 2015; Barbonetti *et al.*, 2016; Lu *et al.*, 2017).

Where is the definitive antioxidant trial?

If oxidative stress is such an important cause of male infertility then it would seem reasonable to propose that antioxidants should be part of the cure. In animal models exhibiting infertility as a consequence of oxidative stress (the GPx5 knock-out mice and a testicular heating model; Chabory *et al.*, 2009; Aitken, 2009; Pérez-Crespo *et al.*, 2008) there is irrefutable evidence that antioxidants can effectively reverse the consequences of oxidative stress in the male germ line *in vivo* (Gharagozloo and Aitken, 2011; Gharagozloo *et al.*, 2016). Similarly, *in vitro*, there is abundant evidence to indicate that antioxidants can reverse the negative impact of oxidative stress sustained during the cryostorage of spermatozoa in a large number of disparate species including the stallion (Ghallab *et al.*, 2017), ram (Gastal *et al.*, 2017), bull (Eidan, 2016) goat (Seifi-Jamadi *et al.*, 2017), rooster (Partyka *et al.*, 2017), rabbit (Zhu *et al.*, 2017), man (Thomson *et al.*, 2009; Parameswari *et al.*, 2017), boar (Gadani *et al.*, 2017), buffalo (Longobardi *et al.*, 2017), dog (Setyawan *et al.*, 2016) and mouse (Chen *et al.*, 2016). However, data to support the use of oral antioxidant therapy to treat human male infertility has been frustratingly difficult to produce (Suleiman *et al.*, 1996). A clinical trial of antioxidant effectiveness would naturally need to be placebo-controlled and should involve the selection of patients on the basis of oxidative stress in their spermatozoa. In order to select such patients a variety of biomarkers might be employed targeting such processes as lipid peroxidation (malondialdehyde, 4-hydroxynonenal, 4-hydroxyhexanal, acrolein) oxidative DNA damage (8OHdG), protein carbonyl formation and ROS production. Moreover, the success of the treatment should be evaluated in terms of improvements in the selected marker

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of oxidative stress as well as aspects of sperm function that are known to be vulnerable to oxidative attack including motility (Jones *et al.*, 1979; Aitken *et al.*, 1989a,b) and sperm–egg recognition (Bromfield *et al.*, 2015). Pregnancy is, of course, an important outcome (Showell *et al.*, 2014), however, there are many factors contributing to a successful outcome in this regard not just the quality of the spermatozoa. In a perfect world we would also look, not just at live pregnancy rates, but also at the mutational load carried by the offspring.

Final thoughts

Male infertility is an aspect of human reproduction desperately in need of attention. It is a highly prevalent condition, affecting around 10% of the male population according to a recent survey (Datta *et al.*, 2016). Moreover, it has relevance not just for fertility but also for the health and wellbeing of future generations. Yet, after decades of study, the underlying aetiology of this condition is still shrouded in uncertainty. Oxidative stress appears to be a major component of the male infertility landscape and, importantly, a mechanism by which extrinsic environmental and lifestyle factors can influence not just the fertility of individuals but also the health of their children. However, it is not the only factor. Spermatogenesis is an inherently complex, highly integrated system that can become compromised for a variety of genetic and epigenetic reasons that have nothing to do with oxidative stress (Jamsai and O'Bryan, 2011; Flannigan and Schlegel, 2017; Jenkins *et al.*, 2017). Some of these we know about (Y-chromosome deletions, Kallmann syndrome, primary ciliary dykinesias, globozoospermia, protamine deficiencies, mutations in CFTR, etc.) and involve defects in known genes. However, it is probable that many cases of infertility do not involve mutations in the coding areas of the genome but lesions in non-coding regions that affect the timing and magnitude of gene expression rather than the functional integrity of the proteins they encode. Whichever regions of the genome are involved, recent data clearly suggest that male infertility has a heritable component that assisted reproductive technologies serve to perpetuate, ensuring that the infertility affecting the fathers of ICSI children will be revisited upon their sons (Belva *et al.*, 2016). A major task that now awaits our attention is to define the relative contribution of such genetic/epigenetic factors and oxidative stress in both the causation of male infertility and the long-term health trajectory of the offspring. The green shoots of discovery are certainly beginning to appear but, for the moment, desideration and achievement in this area are standing on opposite banks of a river deep in misunderstanding and neglect.

Author's roles

R.J.A. conceived, wrote and edited this review.

Conflicts of interest

R.J.A. holds equity in a company, Memphasys, focusing on the development of novel technologies for the isolation of spermatozoa exhibiting low levels of DNA damage. He is also an honorary consultant for CellOxess, a company dedicated to the generation of an antioxidant formulation (Fertilix®) for treating male infertility. R.J.A. also holds a patent, for which the intellectual property is held by Memphasys.

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