REPRODUCTION
REVIEW

Impact of oxidative stress on male and female germ cells: implications for fertility

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Abstract

Male and female germ lines are vulnerable to oxidative stress. In spermatozoa, such stress triggers a lipid peroxidation cascade that culminates in the generation of electrophilic lipid aldehydes that bind to DNA and a raft of proteins involved in the delivery of functionally competent cells. One set of targets for these aldehydes are the proteins of the mitochondrial electron transport chain. When this interaction occurs, mitochondrial ROS generation is enhanced leading to the sustained generation of oxidative damage in a self-perpetuating cycle. Such damage affects all aspects of sperm function including motility, sperm-egg recognition, acrosomal exocytosis and sperm-oocyte fusion. Oxidative stress in the male germ line also attacks the integrity of sperm DNA with potential impacts on the developmental capacity of embryos and the health and wellbeing of the offspring. Potential pathways of reactive oxygen species (ROS) generation in male germ cells could involve enhanced lipoxygenase activity, activation of NADPH oxidase and/or electron leakage from mitochondria. Similarly, in the female germ line, both the induction of oocyte senescence following ovulation and the deterioration of oocyte quality with maternal age appear to involve the generation of oxidative damage. In this case, the mitochondria appear to be a particularly important source of ROS compromising the viability and fertilizability of the oocyte and interfering with the normal segregation of chromosomes during meiosis. In light of these considerations, antioxidants should have some role to play in the preservation of reproductive function in both men and women; however, we still await appropriate trials to test this hypothesis.

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Introduction

Redox reactions are an essential component of living systems and exist in a state of dynamic equilibrium whereby the loss (oxidation) and gain (reduction) of electrons are carefully balanced. Oxidative stress is created when the generation of reactive oxygen species (ROS) by a given cell, tissue or fluid exceeds the protective capacity of the intrinsic antioxidant mechanisms. ROS, in this context, refers to any molecule or compound capable of oxidizing biological substrates including fats, proteins and DNA and may come in the form of free radicals characterized by unpaired valency electrons (such as superoxide anion [$O_2^{-\cdot}$] generated by the one electron reduction of molecular oxygen) or powerful oxidants (such as hydrogen peroxide [H_2O_2] or peroxynitrite [$ONOO^-$]). Antioxidants, on the other hand, come in the form of enzymes that metabolize potentially toxic ROS such as hydrogen peroxide (the glutathione peroxidase system or catalase) and $O_2^{-\cdot}$ (e.g. superoxide dismutase or, possibly, indoleamine dioxygenase) or small molecular mass scavengers that can terminate free radical-mediated chain reactions (such as vitamins C and E or a variety of polyphenols). Some proteins (e.g. lactoferrin) or small molecules (e.g. penicillamine) can also provide antioxidant protection by chelating transition metals that, by redox cycling, can catalyse free radical-mediated reactions.

These pro- and anti-oxidant systems normally exist in a state of balance whereby biologically important oxidation reactions are allowed to occur in a carefully controlled manner. However, if this balance is perturbed because of a lack of antioxidant protection or an overproduction of ROS, then a state of imbalance is created and substrates become oxidized in a unregulated manner leading to pathological change. Some of the products created by such random acts of oxidative vandalism may also be cytotoxic and exacerbate the state of stress. For example, electrophilic lipid aldehydes (such as 4-hydroxynonenal [4HNE] or acrolein) generated as a consequence of lipid peroxidation can themselves bind to a wide variety of biologically important targets, such as the nucleophilic centres of proteins and modify the function of these molecules in a way that powerfully reinforces the pathological impact. For example, lipid

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aldehydes can bind to proteins in the mitochondrial electron transport chain including ATP synthase subunit β (ATP5B), succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHA) and NADH dehydrogenase [ubiquinone] iron–sulfur protein 2 (NDUFS2) (Zong & Yin 2015). As a result of this interaction the electron transport process becomes disorganised, leading to the leakage of electrons that are immediately swept up by the universal electron acceptor, oxygen, to generate $O_2^{-\cdot}$; the latter then rapidly dismutates to hydrogen peroxide, which promotes further lipid peroxidation thereby propagating the state of oxidative stress (Aitken *et al*. 2012). Such mechanisms are capable of creating oxidative damage in a variety of biological systems and are thought to underpin a wide range of pathologies including Parkinson's disease, cancer, artherosclerosis, heart failure, Alzheimer's, depression and ageing. However, there are few systems as vulnerable to oxidative attack as the reproductive system.

Oxidative stress has been repeatedly implicated in the aetiology of male infertility (Bisht *et al*. 2017, Aitken 2018), including the reproductive toxicity that flows from exposure to environmental toxicants (Kovacic & Jacintho 2001) and electromagnetic radiation (Houston *et al*. 2016) as well as clinical conditions such as varicocoele (Agarwal *et al*. 2009) and testicular torsion (Vaos & Zavras 2017). Oxidative stress has also been implicated in the aetiology of female infertility, including such conditions as polycystic ovary syndrome, endometriosis, premature ovarian failure and preeclampsia (Lu *et al*. 2018, Park *et al*. 2018). In addition, oxidative stress has been linked with the negative impact of repeated ovarian stimulation on reproductive competence (Chao *et al*. 2005) as well as the developmental potential of oocytes in an IVF setting (Das *et al*. 2006) or in response to ageing (Lord & Aitken 2013, Mihalas *et al*. 2017). Following the establishment of pregnancy, this form of stress can again impact the functionality of the placenta and ultimately the health and wellbeing of the foetus, with implications for the aetiology of miscarriage, premature rupture of the membranes and foetal death (Burton & Jauniaux 2011, Maiti *et al*. 2017, Domínguez-Perles *et al*. 2019).

A detailed consideration of the role of oxidative stress in the causation of reproductive disorders is of importance because it has clear implications for the prevention, diagnosis and therapeutic resolution of such conditions. In this review, we shall consider evidence supporting a role for oxidative stress in the pathophysiology of reproductive disease with particular emphasis on the male and female germ line.

Oxidative stress in the male

Precursor germ cells

One of the first reproductive functions shown to be vulnerable to oxidative stress was male fertility. We have known that the male germ line is vulnerable to oxidative stress since the sterilizing impact of ionizing irradiation on male fertility was discovered in the 1930s (Snell & Aebersold 1937, Ogilvy-Stuart & Shalet 1993). The differential sensitivity of testicular germ cells to the free radicals generated by ionizing radiation is also indicated by the powerful protective action of antioxidants such as melatonin (Hussein *et al*. 2006) and has been exploited in the extensive use of radiation therapy to successfully treat testicular germ cell tumours (Rajpert-De Meyts *et al*. 2016). The vulnerability of precursor germ cells to oxidative stress is also reflected in the reproductive consequences of testicular heating. Thus, for the testes to be fully functional, they have to operate at a degree or two lower than core body temperature. Heating the testes to 40–41°C induces a rapid loss of germ cells at the pachytene spermatocyte and spermatid stages of differentiation via a combination of Fas-mediated apoptosis and autophagy (Zhang *et al*. 2012). The loss of fertility observed under these circumstances can be completely reversed by the direct administration of antioxidants or treatments that elevate the levels of antioxidant protection within the testes (Gharagozloo *et al*. 2016, Kumar Roy *et al*. 2016). The role of oxidative stress in mediating the effects of testicular heating has also been indicated in studies involving male germ cells from SOD-1-knockout mice which were shown to be more vulnerable to heat stress than controls, *in vitro* and *in vivo* (Ishli *et al*. 2005). Testicular germ cells are thus vulnerable to oxidative stress and none more so than round spermatids, which are particularly vulnerable to lipid peroxidation and the cytotoxic consequences of lipid aldehyde generation (Bromfield *et al*. 2017). It has been proposed that this particular stage of germ cell development is vulnerable to a form of cell death known as ferroptosis, which is dependent on iron and driven by the peroxidative degradation of lipids and accumulation of electrophilic aldehydes such as 4-HNE (Bromfield *et al*. 2019). Furthermore, oxidative stress at the spermatid stage is thought to underpin the subsequent appearance of functional defects in the spermatozoa particularly their competence for sperm-egg recognition (Bromfield *et al*. 2017).

Spermatozoa

The central role of oxidative stress in regulating the functionality of spermatozoa has been recognized since the pioneering studies of Tosic and Walton on the susceptibility of bovine spermatozoa to hydrogen peroxide (Tosic & Walton 1946, 1950). John MacLeod similarly observed the susceptibility of human spermatozoa to hydrogen peroxide stress following the incubation of these cells for prolonged periods of time in high oxygen-tension media (MacLeod 1943). Indeed, spermatozoa were the first cell type in which the metabolic generation of cytotoxic quantities of hydrogen

peroxide was indicated, preceding the discovery of ROS generation by phagocytic leukocytes by several decades (McLeod 1943, Babior *et al.* 1973). The protective effect of catalase on sperm motility, demonstrated initially by McLeod (1943) and substantiated by Aitken *et al.* (1993), strongly suggests that the primary cytotoxic ROS is hydrogen peroxide generated by the dismutation of $O_2^{-\cdot}$ (Alvarez *et al.* 1987). These observations raise fundamental questions about the sources of ROS driving male infertility as well as their mechanisms of action, functional significance and potential remediation.

Sources of ROS in sperm – amino acid oxidase

The biochemical sources of the ROS generated by mammalian spermatozoa have still not been fully resolved but are clearly multiple (Aitken *et al.* 2003) and vary significantly between species (Fig. 1). The first source to be identified was an amino acid oxidase in bovine spermatozoa (Tosic & Walton, 1950). This oxidase utilizes aromatic amino acids as substrate (tyrosine, tryptophan and phenylalanine) and generates hydrogen peroxide and ammonia in the presence of oxygen. An interesting feature of this system is that its contribution to the redox status of bovine sperm was insignificant until the spermatozoa were immersed in an egg-yolk-based extender, in preparation for cryostorage. At this moment, the free aromatic amino acids present in egg yolk stimulated such high levels of ROS generation that motility was curtailed. It was subsequently demonstrated that the simple addition of isolated aromatic amino acids, such as phenylalanine or tyrosine, to suspensions of bovine spermatozoa was enough to suppress their motility and, ultimately, their vitality (Lapointe & Sirard 1998). Interestingly the source of the ROS was traced to the dead cells in the ejaculate which, because of

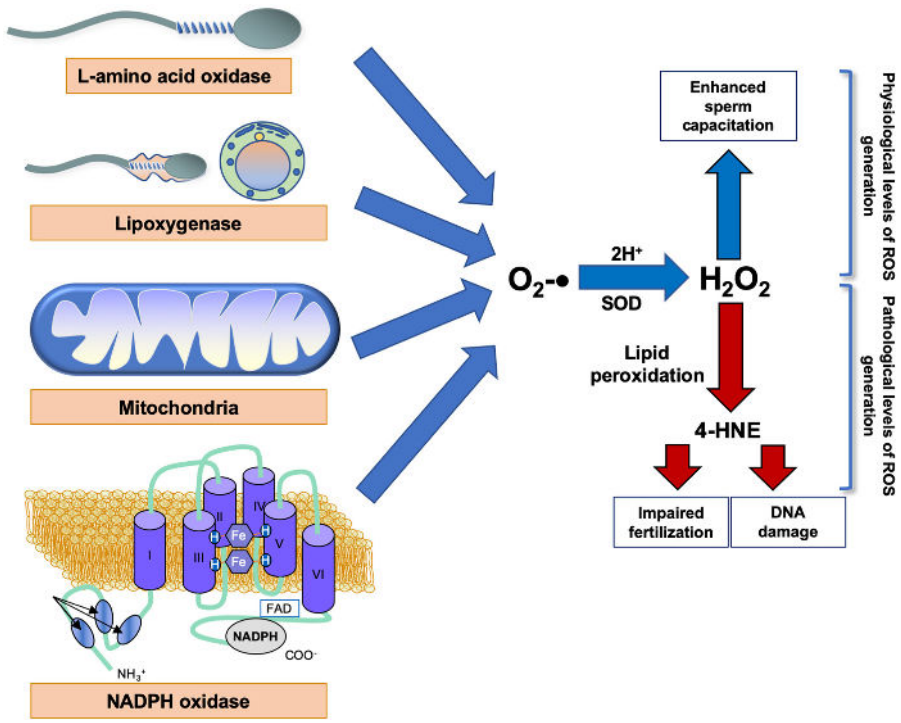

Figure 1 Possible mechanisms by which oxidative stress can be created in spermatozoa. The spermatozoa of several species are known to possess an L-amino oxidase that uses aromatic amino acids as substrate (Houston *et al.* 2015). The biological purpose of these oxidases is not known with certainty; however, they are a significant source of damaging ROS in domestic animals (bull, stallion and ram) exposed to high concentrations of aromatic amino acids in egg yolk-based cryopreservation media. Lipoxygenase is another potential source of ROS, the presence of which may reflect the retention of excess residual cytoplasm as a consequence of defective spermiogenesis and the presence of excess free unesterified free fatty acids (Walters *et al.* 2018). Lipoxygenase may also be a source of ROS in round spermatids by triggering a caspase-independent form of apoptosis known as ferroptosis (Bromfield *et al.* 2019). The mitochondria are a major source of ROS in spermatozoa and are heavily involved in the induction of senescence and apoptosis (Koppers *et al.* 2011). Many species, not mouse, also possess an NADPH oxidase (NOX5) that is capable of generating ROS in a calcium-dependent manner. Overexpression of this enzyme has been linked to the loss of sperm motility observed in asthenozoospermia (Vatannejad *et al.* 2019). The major product from all of these sources is $O_2^{-\cdot}$ which then dismutates to H_2O_2 under the influence of superoxide dismutase (SOD). H_2O_2 is a powerful membrane permeant oxidant that when generated in moderate amounts stimulates sperm capacitation. However, in excess, this oxidant attacks unsaturated fatty acids in the plasma and mitochondrial membranes to induce a lipid peroxidation cascade culminating in the genesis of small molecular mass electrophilic aldehydes such as 4-HNE. The latter then binds to nucleophilic centres within DNA and proteins, inducing a range of pathological challenges to the functionality of spermatozoa and the integrity of their genetic cargo.

their ruptured plasma membranes, were able access the amino acid substrates that fuel the oxidase's ROS-generating activities. In light of these results, the semen extenders used for bovine sperm cryostorage have been reconfigured to contain catalase, to scavenge the hydrogen peroxide emanating from dead spermatozoa in the ejaculate before the motility and vitality of the live cells could become compromised (Shannon & Curson 1982). Similarly, in ram and equine spermatozoa an aromatic amino acid oxidase has been found with similar attributes to the bovine enzyme. Thus, non-viable ram and equine spermatozoa respond to exogenous aromatic amino acids such as L-phenylalanine with a significant increase in ROS generation that then suppresses the total and progressive motility in the live sperm population (Upreti *et al*. 1998, Aitken *et al*. 2015*a*). Importantly the D-forms of these amino acids are incapable of stimulating ROS generation by mammalian spermatozoa; only the L-amino acids are active in this system.

Human spermatozoa also possess an amino acid oxidase with a preference for aromatic amino acids particularly phenylalanine and tryptophan. The L-amino oxidase enzyme in human spermatozoa is encoded by interleukin (IL)-4-induced gene1 (IL4I1), and the gene product is located in the head and midpiece of the cell (Houston *et al*. 2015). In contrast to other species, the enzyme is not retained by dead cells that have lost integrity in their plasma membranes. As a result, non-viable spermatozoa do not respond to aromatic amino acid stimulation and so the threat posed by cryopreservation media supplemented with egg yolk does not apply in the case of human spermatozoa (Houston *et al*. 2015).

The functional significance of this amino acid oxidase is the subject of ongoing discussion but might be related to the induction of sperm capacitation. This process is known to be redox regulated, driven by the ability of ROS to effect a number of critical changes in these cells, including cholesterol oxidation, activation of cAMP generation and inhibition of tyrosine phosphatase activity (Aitken & Curry 2011, Brouwers *et al*. 2011). Exposure to exogenous phenylalanine will induce such changes via mechanisms that can be reversed by the concomitant presence of catalase, suggesting that hydrogen peroxide is the active ROS in this instance, as suggested by others (Bize *et al*. 1991, Rivlin *et al*. 2004). In keeping with this hypothesis, exogenous phenylalanine does induce capacitation and the acrosome reaction in human spermatozoa (Houston *et al*. 2015). However, the quantities of phenylalanine required to achieve this effect (5–10 mM) are higher than the physiological concentrations of phenylalanine in human or, even, bovine uterine fluid (Elhassan *et al*. 2001, Kermack *et al*. 2015). Alternative functions for this oxidase include the oxidative deamination of aromatic amino acids, generating keto acids that are then oxidized by the sperm mitochondria. Such a role may be particularly important in stallion spermatozoa which are highly dependent on oxidative phosphorylation to meet their energy needs and relatively resistant to oxidative stress (Griffin *et al*. 2019). This rationale may also apply to the spermatozoa of other ungulates such as the boar and bull (Storey 2008) which are also heavy users of oxidative phosphorylation. However, this explanation does not apply to human spermatozoa which are primarily driven by glycolysis (Calvert *et al*. 2019). Yet another possibility is that the human oxidase, IL4I1, may be part of an ancient bactericidal system, as a consequence of the generation of ammonia and hydrogen peroxide. IL4I1 may also limit T-lymphocyte activation and proliferation, in part, via the production of hydrogen peroxide and local phenylalanine depletion. The diminished T cell activation would, in turn, lead to impaired proliferation and a disrupted capacity to produce Th1 cytokines and proinflammatory chemokines, creating a state of immunotolerance towards these potentially immunogenic cells (Castellano & Molinier-Frenkel 2017).

Sources of ROS in sperm – mitochondria

Another potential source of ROS, particularly in moribund or senescent spermatozoa, are the mitochondria (Koppers *et al*. 2008; Fig. 1). Human spermatozoa are unusual in that while they may possess mitochondria in the midpiece, they normally rely heavily on the oxidation of substrates via the glycolytic pathway to meet their energy demands. Moreover, human sperm mitochondria are highly prone to electron leakage, leading to ROS generation and the suppression of sperm function. A variety of factors are known to promote ROS generation by human sperm mitochondria, some directly, by interfering with the regulated passage of electrons along the electron transport chain, such as radiofrequency electromagnetic radiation (Houston *et al*. 2018) or polyunsaturated fatty acids (Aitken *et al*. 2006) and others indirectly by triggering the intrinsic apoptotic pathway, such as parabens or bisphenol A (Barbonetti *et al*. 2016, Samarasinghe *et al*. 2018).

The unregulated leakage of electrons from human sperm mitochondria is capable of causing extensive oxidative harm to these cells including the induction of significant DNA damage, lipid peroxidation and motility loss (Houston *et al*. 2018). Clinically, the excessive generation of ROS by human sperm mitochondria has been linked to defective sperm function including the loss of motility seen in asthenozoospermia patients (Cassina *et al*. 2015, Nowicka-Bauer *et al*. 2018). In principle, electron leakage can occur at either Complex 1 or Complex III, although the former seems to be the most damaging. This is because ROS generation at Complex III leads to the rapid release of hydrogen peroxide into the extracellular space, whereas ROS produced at Complex I are released to the matrix where they can induce peroxidative damage to the inner mitochondrial membrane (Koppers *et al*. 2008).

Although the enhanced mitochondrial ROS generation, seen spontaneously in the defective spermatozoa of infertile patients or triggered by cytotoxic reagents, has been linked with a loss of mitochondrial membrane potential (MMP) (Wang *et al.* 2003), this association may be indirect in nature. Thus, it has been found that electron transport inhibitors can readily stimulate mitochondrial ROS generation in human spermatozoa in the absence of any immediate change in MMP (Koppers *et al.* 2008). However, it is very possible that the prolonged generation of ROS from Complex 1 in the immediate vicinity of the inner mitochondrial membrane might secondarily lead to an opening of the transient permeability transition pore and a secondary collapse of the MMP (Zorov *et al.* 2014).

Sources of ROS in sperm – NADPH oxidase and sperm capacitation

The possible involvement of an NADPH oxidase in the generation of ROS by human spermatozoa was suggested by the discovery that the addition of this co-enzyme to suspensions of human spermatozoa could induce a burst of oxidative activity reflected by an increase in lucigenin-dependent chemiluminescence (Aitken *et al.* 1997; Fig. 1). Although reservations have been rightly raised about the specificity of the lucigenin probe as a means of detecting $O_2^{-\cdot}$ (Baker *et al.* 2004, 2005), the same burst of activity was observed when acetylated cytochrome C reduction, a more specific $O_2^{-\cdot}$-detection reagent, was used to monitor the response to NADPH (Aitken *et al.* 1997). Furthermore, the involvement of an NADPH oxidase in the generation of ROS by human spermatozoa has been suggested by the suppressive action of diphenylene iodonium (DPI), a recognized NADPH oxidase inhibitor (Aitken *et al.* 1997). Although DPI is a generalized flavoprotein inhibitor, which is necessarily not specific for NADPH oxidase activity, Donà *et al.* (2011) demonstrated that ROS production by human sperm suspensions could be suppressed by another NADPH oxidase inhibitor, apocynin. Moreover, the generation of ROS by purified suspensions of human spermatozoa was shown to be correlated with intracellular glucose-6-phosphate dehydrogenase, a key determinant of intrinsic intracellular NADPH generation via the hexose monophosphate shunt (Aitken *et al.* 1994). Finally, an NADPH oxidase (NOX5) has been definitively identified in human spermatozoa and shown to generate ROS in a calcium-dependent manner via mechanisms involving the mediation of a non-receptor tyrosine kinase, c-Abl, and the HV1 proton channel (Musset *et al.* 2012).

The NCBI gene database indicates that the gene encoding the NOX5 protein is present in the genome of several mammalian species including cattle, horse, pig, cat, dog, rabbit, chimpanzee, rhesus monkey, armadillo, ferret, opossum and human. However the NOX5 gene does not appear to be represented in the mouse genome. So if NOX5 does, as proposed, play a central role in sperm biology, it is not a ubiquitous mechanism. Nevertheless, from a clinical perspective, asthenozoospermia has been associated with the over-expression of NOX5 compared with normozoospermic males, as well as significantly elevated levels of peroxynitrite, $O_2^{-\cdot}$ and hydrogen peroxide generation (Vignini *et al.* 2006, Vatannejad *et al.* 2019). In view of these data, a potential role for this oxidase in the pathogenesis of defective sperm function cannot be ruled out.

Polyunsaturated fats and lipoxygenase

Another source of ROS within the male germ line is lipoxygenase (Fig. 1). It has been known for some time that polyunsaturated fatty acids can induce ROS formation by human spermatozoa (Aitken *et al.* 2006). In addition to arachidonic acid, this activity could be induced by other cis-unsaturated fatty acids including linoleic and docosahexaenoic acids; however, saturated fatty acids, methyl esters of unsaturated fatty acids or other amphiphiles were all ineffective. The relevance of these findings to male infertility was suggested by the positive correlation that has been observed between the free unsaturated fatty acid content of defective human spermatozoa and their relative capacity for spontaneous mitochondrial $O_2^{-\cdot}$ generation (Koppers *et al.* 2010). The underlying mechanism was postulated to be the disruption of regulated electron flux along the mitochondrial electron transport chain, leading to the leakage of electrons that are then swept up by oxygen to generate $O_2^{-\cdot}$. Another possibility is that polyunsaturated fatty acids somehow promote the opening of the mitochondrial permeability transition pore (mPTP) which, in turn, leads to a loss of mitochondrial membrane potential, followed by electron leakage and $O_2^{-\cdot}$ generation in spermatozoa. The link between polyunsaturated fats such as arachidonic acid and mPTP opening could involve the mediation of hydroxyeicosatetraenoic acids (HETEs) generated via lipoxygenase action. HETEs are known to activate Ca^{2+}-induced opening of the mPTP in, for example, the failing heart, and exposure to lipoxygenase inhibitors has been found to attenuate this process (Moon *et al.* 2018). Under normal circumstances, the unsaturated fat that dominates the sperm lipid profile, decosahexaenoic acid, might be expected to suppress mPTP opening (Khairallah *et al.* 2010). However, in the presence of excess lipoxygenase, unsaturated fatty acids, particularly arachidonic acid, would be expected to stimulate HETE generation, mPTP opening, electron leakage and ROS generation in a chain of cause and effect. Normally, most lipoxygenase activity is lost from mammalian spermatozoa during epididymal maturation when the cytoplasmic droplet is discarded (Fischer *et al.* 2005). However, defective human spermatozoa have been shown to possess excess residual cytoplasm (Gomez

et al. 1996) and, as a consequence, are associated with an elevated cellular content of lipoxygenase activity as well as excess arachidonate (Yu *et al.* 2019). Moreover, suppression of lipoxygenase activity in human spermatozoa has been shown to protect human spermatozoa from the oxidative stress, created following exposure to exogenous hydrogen peroxide (Walters *et al.* 2018). A possible interpretation of these data would be that (1.) hydrogen peroxide exposure directly induces lipid peroxidation; (2.) major by-products of the lipid peroxidation cascade include lipid aldehydes that bind to redox centres within the mitochondrial electron transport chain stimulating the regeneration of sustained high levels of ROS; (3.) sustained oxidative stress leads to mitochondrial calcium overload; (4.) elevated intra-mitochondrial calcium levels then lead to the stimulation of PLA2 activity which induces an increase in free arachidonate and lipoxygenase-mediated HETE generation and (5.) the HETEs promote mPTP opening, a consequential collapse of mitochondrial membrane potential, ATP depletion, yet more ROS generation and finally induction of the intrinsic apoptotic cascade and creation of DNA damage (de Lamirande & Gagnon 1992, Peng & Jou 2010, Koppers *et al.* 2011). Such a cascade is consistent with the clear linkages that have been reported on many independent occasions between impaired sperm function and elevated mitochondrial ROS generation, lipid peroxidation, intracellular calcium overload, mPTP opening, loss of mitochondrial membrane potential and DNA damage (Aitken & Curry 2011, Zhang *et al.* 2014). Clearly, further studies will have to be conducted to determine the details of how all of these various factors intersect.

Redox regulation of sperm function – the yin and the yang

Whilst it is becoming clear that excess ROS generation by spermatozoa is involved in the aetiology of defective sperm function, this leaves open the question as to why spermatozoa would evolve systems for the generation of ROS in the first place; particularly when the vulnerability of these cells to oxidative stress is so apparent. The answer to this question appears to be that a key aspect of sperm physiology, namely their ability to capacitate, is redox regulated.

In this context, it has been repeatedly shown that capacitation has been linked to the cellular generation of ROS (Bize *et al.* 1991, de Lamirande & Gagnon 1993, Rivlin *et al.* 2004, Aitken *et al.* 2015*b*) which is, in turn, dependent on the presence of bicarbonate and cytoplasmic alkalinization (Ecroyd *et al.* 2003). Furthermore, the ROS-generating capacity of spermatozoa can be reduced by DPI, high concentrations of Zn and semenogelin, all of which suppress sperm capacitation (Ecroyd *et al.* 2003, de Lamirande & Lamothe 2010). Conversely, the Zn chelator, TPEN, enhances $O_2^{-\cdot}$ anion generation and stimulates sperm capacitation (de Lamirande *et al.* 2009). Progesterone, a physiological promoter of sperm capacitation and acrosomal exocytosis, has also been found to stimulate ROS generation (Ghanbari *et al.* 2019). Moreover, ROS generation stimulates cAMP generation, cholesterol oxidation and tyrosine phosphorylation, all of which are associated with the attainment of a capacitated state (White & Aitken 1989, Aitken *et al.* 1998*b*, Lewis & Aitken 2001, Aitken & Nixon 2013).

An additional contribution to the physiological induction of sperm capacitation may also be made by another radical species, nitric oxide (NO), which can induce many of the hallmarks of sperm capacitation including an upregulation in phosphotyrosine expression, the stimulation of hyperactivated movement and the enhancement of sperm-zona interaction (Zini *et al.* 1995, Sengoku *et al.* 1998, Herrero *et al.* 1999). Furthermore, a lack of NO stimulation appears to be associated with sperm pathologies of various kinds (Kalezic *et al.* 2018). Just as we saw with $O_2^{-\cdot}$, Zn inhibitors and Zn chelators promote both NO generation by human spermatozoa and capacitation (de Lamirande *et al.* 2009). Moreover, seminogelin, the major protein constituent of the human semen coagulum, blocks capacitation via the suppression of both $O_2^{-\cdot}$ and NO generation (de Lamirande & Lamothe 2010). Exposure of human spermatozoa to NO sources has also been shown to promote the tyrosine phosphorylation events associated with capacitation, while nitric oxide synthase inhibitors have been found to suppress this process (Herrero *et al.* 1999). The rapid reaction between NO and $O_2^{-\cdot}$ anion creates the powerful oxidant, peroxynitrite ($ONOO^-$). Peroxynitrite modifies tyrosine in proteins to create nitrotyrosines, leaving a detectable footprint. Human spermatozoa have been shown to express nitrotyrosine residues suggesting that peroxynitrite, a strong oxidizing agent, may well be a physiological inducer of the oxidative changes associated with sperm capacitation *in vivo*, including both hyperactivation and the sperm-zona binding competence of these cells (Herrero *et al.* 2001). The generation of such a powerful oxidant during sperm capacitation will also inevitably place limits on the long-term survival of spermatozoa once they have engaged the capacitation process. Indeed, following this line of logic, it has been suggested that sperm capacitation and senescence are opposite ends of a biological continuum driven by peroxynitrite (Aitken *et al.* 2015*b*).

In light of the foregoing, it is clear that reactive oxygen and nitrogen species are two-edged swords as far as spermatozoa are concerned. On the one hand, these reactive metabolites are critical to the cascade of oxidative events driving sperm capacitation by virtue of their capacity to stimulate cAMP generation, impair tyrosine phosphatase activity and enhance oxysterol formation. On the other, over-capacitation of spermatozoa leads to a state of senescence and the activation of a truncated

intrinsic apoptotic cascade characterized by enhanced mitochondrial ROS generation, lipid oxidation, motility loss, caspase activation and phosphatidylserine externalization (Aitken *et al.* 2015*b*). This truncated apoptotic cascade constitutes a regulated biological mechanism that allows spermatozoa that are surplus to requirements to die in a carefully controlled manner. This is important because it permits the immune system to phagocytose senescent spermatozoa without triggering an unwanted inflammatory response. This situation arises by virtue of the fact that apoptotic cells, expressing phosphatidylserine on their surface, are able to induce a 'silent' phagocytosis on behalf of the neutrophils and macrophages entering the lower female reproductive tract following insemination (Aitken & Baker 2013). Levels of oxidative stress that are not sufficient to induce cell death via apoptosis are still capable of disrupting all aspects of sperm function including motility, sperm-zona recognition, acrosomal exocytosis and sperm-oocyte fusion. In addition, sublethal levels of oxidative stress are known to impact the integrity of sperm DNA thereby influencing the potential development of the embryo and the health and wellbeing of the offspring (Aitken *et al.* 1998*a*, Aitken & Curry 2011, Aitken 2018). For example, the altered patterns of gene expression observed in equine embryos as a consequence of insemination with cryostored spermatozoa maybe a case in point, because cryostorage is known to be associated with the induction of oxidative DNA damage (Thomson *et al.* 2009, Ortiz-Rodriguez *et al.* 2019).

Oxidative stress in the female

The oocyte

The female germ line is as vulnerable to oxidative stress as the male. Just as oxidative stress plays a key role in the senescence of spermatozoa post ejaculation, such stress appears to be central to the mechanisms by which the oocyte loses its developmental competence post ovulation. In this context, oocytes are actually more vulnerable than spermatozoa. Within a few hours of ovulation, the oocyte loses its viability and functionality, while ejaculated spermatozoa are designed to spend several days in the female reproductive tract waiting for an egg to arrive; particularly in our own species where the acts of insemination and ovulation are not synchronized by the expression of oestrus. Oocytes are therefore programmed to die rapidly after ovulation unless they are rescued by union with a spermatozoon – whereupon they become (potentially) immortalized.

The mechanism by which postovulatory oocytes enter apoptosis and lose their functionality involves a complex series of events, all of which are driven by an increase in oxidative stress. For example, zona pellucida hardening is a classic feature of post-ovulatory oocyte ageing which can be driven by exposure to $O_2^{-\cdot}$ and involves the cross linking of tyrosine residues. This oxidative event is catalysed by an ovoperoxidase located in cortical granules which are released from the oocyte surface in an exocytotic process which is facilitated by ageing and is, again, driven by $O_2^{-\cdot}$ (Hoodbhoy & Talbot 1994, Goud *et al.* 2008). Oxidative stress also appears to be involved in the non-dysjunction of chromosomes that characterizes oocytes that have aged *in vivo* and is responsible for the exponential increase in aneuploidy in the embryos conceived by woman over the age of 35 (Tarin *et al.* 1998).

In a manner reminiscent of senescent spermatozoa, oxidative stress during the post-ovulatory ageing of oocytes is created by the leakage of electrons from the oocyte mitochondria driving a lipid peroxidation process that culminates in the generation of toxic lipid aldehydes such as 4-HNE and acrolein (Lord *et al.* 2015, Jeelani *et al.* 2018). These aldehydes, in turn, mediate a great deal of the cellular damage that ultimately induces the post-ovulatory apoptotic demise of the oocyte including the induction of mitochondrial ROS generation, a loss of mitochondrial membrane potential, DNA damage and activation of the intrinsic apoptotic cascade. A major target for these electrophilic aldehydes again appears to be proteins within the mitochondrial electron transport chain such as succinic acid dehydrogenase. Adduction of this enzyme by lipid aldehydes such as acrolein or 4-HNE disrupts electron flow within the mitochondria, promoting electron leakage and ROS generation, that then reinforce the creation of oxidative stress in a positive feedback loop. As a result of this chemistry, the oxidative attack becomes sustained, initiating a cascade of events that create the aged-oocyte phenotype (Lord & Aitken 2013).

Remarkably, the act of fertilization abruptly halts this self-induced, post-ovulatory decline in viability; however, how this is achieved is currently unknown. We do know that fertilization is followed by a sudden increase in the DNA-repair capacity of the oocyte such that the latter is significantly more resistant to the ability of etoposide to elicit double strand breaks in oocyte DNA. This change is at least partly mediated by the fertilization-dependent upregulation of permeability glycoprotein (PGP), an endogenous multidrug efflux transporter that is translocated to the oolemma and phosphorylated upon oocyte activation, thereby enhancing the ability of the oocyte to remove compounds that might compromise DNA integrity (Martin *et al.* 2016). Many other changes are invoked in the oocyte following fertilization, not least the activation of calcium oscillations by sperm-specific phospholipase C. These transients are clearly critical for arresting the tendency of oocytes to descend into an apoptotic decline while activating embryonic development (Swann & Lai 2016). However, the precise mechanism by which these calcium oscillations so dramatically alter the downward, developmental trajectory of the oocyte have not yet been resolved.

Similar oxidative processes appear to mediate the impact of chronological age on oocyte function. In this context, oocytes recovered from ageing female mice are characterized by significantly elevated levels of ROS generation and lipid aldehyde formation as well as a high tendency for chromosome non-dysjunction (Lord *et al.* 2015, Mihalas *et al.* 2017). One of the targets for these electrophilic aldehydes appears to be the proteins involved in the control of chromosome segregation during meiosis (Mihalas *et al.* 2017). Significantly, the age-dependent increase of chromosomal non-dysjunction seen in *Drosophila* oocytes can be completely reversed by the engineered overexpression of SOD 1 and SOD 2 during meiotic prophase. Such studies support the notion that the exponential increase in oocyte aneuploidies seen in ageing women is related to an age-induced increase in oxidative stress that might be amenable to correction through the judicious administration of exogeneous antioxidants (Perkins *et al.* 2019).

An interesting question which is yet to be addressed is whether there are other sources of ROS generation in oocytes. These cells are apparently rich in NADPH oxidase enzymes (Maru *et al.* 2005) and, in some species at least, strong lipoxygenase activity has been detected in the oocyte, with a potential role in oocyte maturation (Hawkins & Brash 1987). However, the contribution of such systems in the biology and pathology of the oocyte is currently unknown (Fig. 2).

Antioxidants

In light of the important role that oxidative stress plays in the aetiology of defective sperm and oocyte function, it is possible that antioxidants may have a therapeutic role to play in the context of both *in vitro* fertilization outcomes and in controlling the impact of age on fertility. *In vitro*, a variety of antioxidants have been shown to enhance the fertilizability of oocytes following *in vitro* maturation. For example, the presence of sodium selenite in the medium has been shown to significantly increase the *vitro* maturation of mouse oocytes while suppressing the generation of ROS and increasing mtDNA copy number (Ghorbanmehr *et al.* 2018), presumably through the stimulation of glutathione peroxidase activity (a key antioxidant enzyme with selenium in its active site). Positive impacts on the *in vitro* maturation of mammalian oocytes have also been recorded for α-tocopherol, either alone (Arias-Álvarez *et al.* 2018) or mixed with additional antioxidants (alpha-lipoic acid, hypotaurine and N-acetyl cysteine) (Pasquariello *et al.* 2019), 9-cis-retinoic acid (Gad *et al.* 2018), coenzyme Q10 (Heydarnejad *et al.* 2019) melatonin (An *et al.* 2019), rosmarinic acid (Zhang *et al.* 2019) and the antioxidant citrus flavonoid, hesperetin (Kim *et al.* 2019). Antioxidants have also been shown to ameliorate the oxidative stress associated with oocyte vitrification (Trapphoff *et al.* 2016, Wang *et al.* 2018, Ahmadi *et al.* 2019) repeated superovulation (Xiao *et al.* 2019), induced polycystic ovarian disease (Eini *et al.* 2019) post-ovulatory oocyte ageing (Liang *et al.* 2018), exposure to heat stress (Cavallari *et al.* 2019), old age (Liu *et al.* 2013) or a variety of toxicants *in vitro* such as menadione, bisphenol A (Cavallari *et al.* 2019, Li & Zhao 2019), Fenoxaprop-ethyl (He *et al.* 2019) and busulfan/cyclophosphamide chemotherapeutic combinations (Wu *et al.* 2019).

Oxidative stress has also been suggested as a detrimental factor in the determination of ICSI success

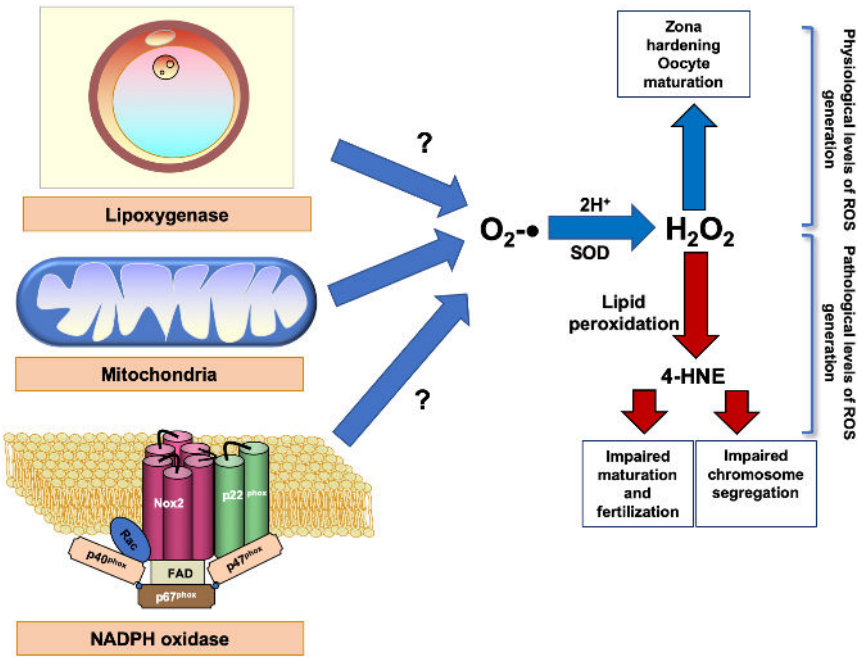

Figure 2 Sources of ROS in oocytes. Much less is known about ROS generation in oocytes. Post-ovulatory oocyte ageing clearly involves ROS generation by the mitochondria (Lord & Aitken 2013). While oocytes possess NADPH oxidase (NOX2) and lipoxygenase, the role of these pathways in creating oxidative stress within the female germ line is unknown. Whatever the origins of the $O_2^{-\cdot}$ generated by these cells, the H_2O_2 produced by dismutation has positive outcomes when the oxidative stimulus is moderate and physiological (zona hardening and oocyte maturation). However negative outcomes (impaired functionality, senescence, DNA damage and impaired chromosome segregation) are observed when ROS are produced in excess (Kala *et al.* 2017). Once again, lipid aldehydes such as, 4-HNE is a key mediator of pathological change.

rates. The results achieved with this form of insemination are enhanced by lowering ambient oxygen tensions (20–5%) or the inclusion of antioxidants in the culture medium such as reduced glutathione (Ashibe *et al*. 2019). Even though high levels of oxidative sperm DNA damage are tolerated by the oocyte in terms of fertilization and pronucleus formation (Twigg *et al*. 1998), it is in the subsequent development of the embryo that the impact of oxidative stress becomes manifest particularly in terms of mitotic arrest after ICSI (Burruel *et al*. 2013). This maybe because of the presence of high levels of unresolved DNA damage leading to the induction of apoptosis or the creation elevated mutational loads as a consequence of aberrant or defective DNA repair. In addition, since the spermatozoon's centromeres are responsible for orchestrating all cell division in the embryo, it is possible that oxidative damage to this subcellular structure in the male gamete results in the impairment of ordered mitosis in the offspring. We have recently found evidence for such an effect in annelid spermatozoa exposed to dibutyl phthalate. This toxicant created a state of oxidative stress in the spermatozoa resulting in the generation of 4-HNE which covalently bound to the sperm centriole, ultimately resulting in disrupted cytoskeletal protein organization during early embryonic cleavage (Lu *et al*. 2017). The extent to which such epigenetic mechanisms may underlie the impact of oxidative stress in the male germ line on the post-fertilization developmental normality of embryos from other species is unknown.

Conclusions

Oxidative stress is clearly a major determinant of functionality in both the male and female germ lines. In the male germ line, there is clear evidence that a low level of ROS generation is beneficial for sperm function, achieving a range of functions from the cross linking of sperm chromatin to the enhancement of sperm capacitation. However, in a variety of situations (cryopreservation, toxicant exposure, age etc.) the generation of ROS becomes super-physiological and overpowers the limited antioxidant defences of these cells precipitating a state of oxidative stress that can impair both sperm production and function and ultimately impact the health trajectory of the offspring. Although we have made some progress in understanding the various sources of ROS in these cells, we are still awaiting a full explanation of the genetic, epigenetic and environmental factors responsible for the oxidative stress seen in subfertile males. In the female germ line, no positive role for ROS has been suggested; however, it is clear that oocytes are vulnerable to the damaging impact of these toxic metabolites if the exposure is intense and/or adequately prolonged. As in the male germ line, cryopreservation, toxicant exposure and ageing, whether *in vitro* or *in vivo*, are all factors in creating pathological levels of oxidative stress in the female germ line with potential impacts on both fertility and the normality of any resulting embryos. Given the susceptibility of reproduction to oxidative stress, antioxidants should be of fundamental significance in preserving the functional integrity of the male and female germ line. Despite the potential inherent in this field and certain islands of promising data, there is little science behind either the precise nature or the dose of antioxidants that have been used to address oxidative stress *in vivo* or *in vitro*. The lack of clinical trials wherein patients are selected for antioxidant therapy based on evidence that their infertility is due to oxidative stress is particularly distressing. Hopefully this deficit will be addressed in the not-too-distant future.

Declaration of interest

The author declares that he is an honorary advisor for CellOxess, a company specializing in the development of antioxidant formulations for the treatment of human infertility.

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