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INVITED REVIEW

Reproductive Health

Oxidative stress and male reproductive health

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One of the major causes of defective sperm function is oxidative stress, which not only disrupts the integrity of sperm DNA but also limits the fertilizing potential of these cells as a result of collateral damage to proteins and lipids in the sperm plasma membrane. The origins of such oxidative stress appear to involve the sperm mitochondria, which have a tendency to generate high levels of superoxide anion as a prelude to entering the intrinsic apoptotic cascade. Unfortunately, these cells have very little capacity to respond to such an attack because they only possess the first enzyme in the base excision repair (BER) pathway, 8-oxoguanine glycosylase 1 (OGG1). The latter successfully creates an abasic site, but the spermatozoa cannot process the oxidative lesion further because they lack the downstream proteins (APE1, XRCC1) needed to complete the repair process. It is the responsibility of the oocyte to continue the BER pathway prior to initiation of S-phase of the first mitotic division. If a mistake is made by the oocyte at this stage of development, a mutation will be created that will be represented in every cell in the body. Such mechanisms may explain the increase in childhood cancers and other diseases observed in the offspring of males who have suffered oxidative stress in their germ line as a consequence of age, environmental or lifestyle factors. The high prevalence of oxidative DNA damage in the spermatozoa of male infertility patients may have implications for the health of children conceived *in vitro* and serves as a driver for current research into the origins of free radical generation in the germ line.

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INTRODUCTION

Male infertility is a relatively common condition affecting approximately 1 in 20 of the male population. In a vast majority of infertile subjects sufficient numbers of spermatozoa are generated to initiate a pregnancy; however, the functionality of the spermatozoa has been compromised. As a result, defective sperm function is held to be the largest, single and defined cause of human infertility.[1] The primary causes of defective sperm function are undoubtedly multifactorial, involving a range of primary genetic, lifestyle and environmental factors, acting alone or, more frequently, in combination. However at the level of the gamete, the integration of these various forces frequently culminates in a state of oxidative stress that impairs the functional and structural integrity of these highly differentiated cells. The first suggestion that oxidative stress might play a role in the etiology of defective sperm function came from one of the pioneers of modern andrology, Dr John MacLeod.[2] He published an important paper in 1943 which demonstrated that in oxygenated medium human spermatozoa rapidly lost motility via mechanisms that could be rescued by the concomitant presence of catalase, a specific scavenger of hydrogen peroxide. The fundamental notion that spermatozoa could generate reactive oxygen species (ROS), specifically hydrogen peroxide, was confirmed by Tosic and Walton in a paper published in Nature in 1946.[3] In this, and a follow-up paper published in 1950,[4] these authors presented impressive biochemical evidence that bovine spermatozoa could not only generate hydrogen peroxide but also that this reactive oxygen metabolite was damaging to sperm function. In this specific case, the authors demonstrated the involvement of an *L*-amino acid oxidase with a particular affinity for aromatic amino acids such as phenylalanine. Many years later Shannon and Curson[5] confirmed the presence of such an oxidase in bovine spermatozoa and demonstrated that it was the dead cells in any given ejaculate that were particularly active in generating hydrogen peroxide in response to phenylalanine and that the oxidative stress generated in this manner could have an impact on the live cells present in the immediate vicinity. The cytotoxic effect of ROS generated on exposure to the phenylalanine in cryostorage medium could be rescued by the concomitant presence of catalase, confirming hydrogen peroxide as the cytotoxic principle.

The notion that oxidative stress might also be a factor in the etiology of defective sperm function in our species was advanced independently by Aitken and Clarkson[6] and Alvarez *et al.*[7] in 1987. An important but often overlooked catalyst for this discovery was the development of a technique for objectively measuring sperm function, in the form of the zona-free hamster oocyte penetration assay introduced by another pioneer of modern andrology, Ryuzo Yanagimachi.[8] Up until this point, the field had lacked objective methods for the measurement of human sperm function aside from motility. The hamster oocyte penetration assay provided an objective means of determining the competence of human spermatozoa to capacitate, undergo the acrosome reaction and generate a fusogenic equatorial segment capable of initiating fusion with the vitelline membrane of the oocyte. In the age of intracytoplasmic sperm injection (ICSI), the hamster oocyte model can also provide critical information on the ability of spermatozoa to form a pronucleus.[9–11] When combined with objective methods for assessing sperm motility, this assay has been shown to give a very accurate assessment of the fertilizing potential of human ejaculates.[12,13] One of the interesting results secured with this assay was to demonstrate

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that defective sperm function was evident in infertile men, even when their spermatozoa had been treated with the divalent cation ionophore, A23187 in order to induce an acrosome reaction.[14] This result indicated that whatever the lesions are in defective spermatozoa, they lay downstream of the calcium influx normally triggered when the spermatozoa make contact with the cumulus-oocyte complex.

Such results suggested that there must be some defect in the plasma membrane of functionally compromised human spermatozoa that prevents them from fusing with the vitelline membrane of the oocyte. It was this quest for an explanation of failed membrane fusion in the hamster oocyte assay that led us to the concept that lipid peroxidation was a key factor in the etiology of defective sperm function. Spermatozoa are particularly vulnerable to lipid peroxidation because they contain high concentrations of unsaturated fatty acids, particularly docosahexaenoic acid with six double bonds per molecule.[15] The latter are vulnerable to free radical attack because the carbon hydrogen dissociation energies are lowest at the bisallylic methylene position. As a consequence, the hydrogen abstraction event that initiates lipid peroxidation is promoted, generating a carbon-centered lipid radical that then combines with oxygen to generate peroxyl (ROO•) and alkoxyl (RO•) radicals that, in order to stabilize, abstract hydrogen atoms from adjacent carbons. These chemical reactions create additional lipid radicals that then perpetuate the lipid peroxidation chain reaction, culminating in the generation of small molecular mass electrophilic lipid aldehydes such as 4-hydroxynonenal (4HNE), acrolein and malondialdehyde. Added to this vulnerability, we have shown that sperm mitochondria respond to the presence of free unsaturated fatty acids with a dramatic increase in ROS generation; the greater the level of unsaturation, the greater the level of the stimulatory effect. Esterification of the fatty acid counters this pro-oxidant effect suggesting that it is the amphiphilic properties of these molecules that are central to their ROS-inducing activity, possibly by defining the orientation of the fatty acids in relation to the mitochondrial electron transport chain. In this context, it is significant that defective human spermatozoa possess abnormally high cellular contents of free polyunsaturated fatty acids, the levels of which are positively correlated with mitochondrial superoxide generation.[17]

Thus, defective human spermatozoa are particularly vulnerable to oxidative stress because they contain a superabundance of free unsaturated fatty acids that trigger ROS generation by the sperm mitochondria and induce high levels of lipid peroxidation. To make matters worse, the products of lipid peroxidation in the form of small molecular mass electrophilic aldehydes such as 4HNE or acrolein, are also capable of triggering ROS generation by the sperm mitochondria.[18] This ability of lipid aldehydes generated as a consequence of lipid peroxidation to trigger mitochondrial ROS generation appears to be a function of their capacity to adduct onto proteins in the mitochondrial electron transport chain, such as succinic acid dehydrogenase.[18] As a consequence of these interactions, it is evident that oxidative stress in human spermatozoa is a self-propagating cycle that, once initiated, will inevitably lead to oxidative damage, a loss of functionality and ultimately, cell death (**Figure 1**).

OXIDATIVE STRESS SPERM FUNCTION, DNA INTEGRITY AND CELL DEATH

One of the first functions affected by oxidative stress and lipid peroxidation is sperm motility. Correlations between lipid peroxide formation and sperm movement have been repeatedly observed in a variety of different species.[15,19,20] Experiments involving exposure of mammalian spermatozoa to a variety of ROS using the xanthine oxidase

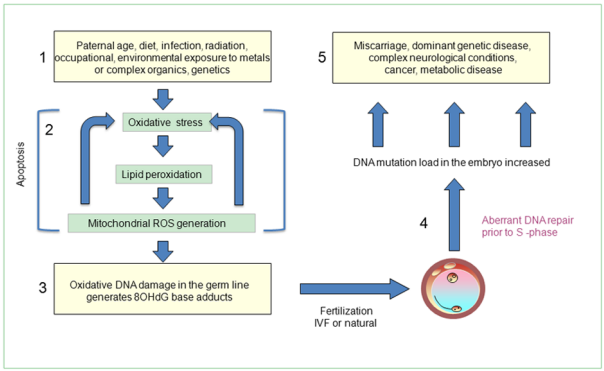

Figure 1: Proposed cycle of cause and effect by which oxidative stress in the male germ line impacts upon the health and well-being of future generations. (1) A variety of primary factors can initiate oxidative stress in the male germ line including infection, age, obesity and exposure to a variety of adverse environmental influences. (2) This initial oxidative stress induces lipid peroxidation culminating in the production of lipid aldehydes such as 4HNE, which bind to proteins in the mitochondrial electron transport chain, stimulating the generation of reactive oxygen species (ROS). The latter stimulate yet more lipid peroxidation in a self-propagating cycle that culminates in apoptosis. (3) One of the most sensitive targets of oxidative stress is the DNA in the sperm nucleus, generating 8-hydroxy, 2'deoxyguanosine (8OHdG) base adducts. (4) The first enzyme in the base excision repair pathway, 8-oxoguanine glycosylase 1 (OGG1), is present in spermatozoa and its activity creates abasic sites. The remainder of the DNA repair pathway is present in the oocyte. The oocyte has to repair the DNA damage brought into the zygote by the fertilizing spermatozoon before the initiation of S-phase for the first mitotic division. (5) If the oocyte makes a mistake at this stage of DNA repair, it has the potential to create a mutation that will be represented in every cell in the body and could account for the range of pathologies seen in the offspring of fathers exhibiting high levels of oxidative DNA damage in their spermatozoa. Abbreviations: IVF, *in vitro* fertilization; ROS, reactive oxygen species.

ROS-generating system have also clearly demonstrated the susceptibility of sperm motility to oxidative attack and identified hydrogen peroxide as the most cytotoxic oxygen metabolite in this context; catalase, but not superoxide dismutase, preventing sperm motility loss under such circumstances.[21–24] The mechanisms by which motility is lost when spermatozoa are under oxidative stress is not known with certainty, but both oxidative damage to the axoneme and depletion of intracellular adenosine triphosphate (ATP) appear to be involved.[25–27]

Notwithstanding the dramatic effects that high levels of exposure to ROS have on sperm motility, it is also evident that oxidative stress can compromise the fertilizing capacity of spermatozoa under conditions where motility is normal.[28,29] Under these circumstances, it is the capacity of the spermatozoa to fuse with the vitelline membrane of the oocyte which is impaired. A careful dose-dependent analysis of the impact of oxidative stress on sperm-oocyte fusion demonstrated a biphasic response which beautifully encapsulates the complex relationship between ROS and sperm function.[30] Thus, at low levels of oxidative stress, sperm-oocyte fusion rates were enhanced, presumably as a consequence of: (i) the positive role that ROS are known to play in driving the tyrosine phosphorylation events associated with sperm capacitation,[31] and (ii) the importance of sterol oxidation in facilitating the efflux of cholesterol from the sperm plasma membrane.[32] However, at higher levels of oxidative stress the induction of lipid peroxidation in the plasma membrane is associated with a decline in the competence for sperm-oocyte fusion, possibly due to the direct induction of oxidative damage to proteins involved in the fusion process, rather than any change in the fluidity of the sperm plasma membrane.[33]

OXIDATIVE STRESS AND DNA DAMAGE

When human spermatozoa were exposed to increasing levels of hydrogen peroxide it was not just the fertilizing potential of the cells that followed a biphasic pattern of change, the DNA in the sperm nucleus behaved similarly. At low levels of oxidative stress DNA damage was diminished, possibly because of the powerful role played by glutathione peroxidase in effecting the cross linking of sperm chromatin. However, at higher levels of oxidative stress, the sperm chromatin started to fragment.[30] Importantly, the losses of fertilizing potential and DNA integrity occurred at different rates, with the latter being the more sensitive. As a result, spermatozoa that had been driven to a high state of readiness for fertilization by low levels of oxidative stress were also found to exhibit significantly elevated levels of DNA damage.[30] This is an extremely significant observation, since it suggests a mechanism by which environmental influences on the paternal germ line could have a major impact on the health trajectory of any progeny.

The oxidized base adduct, 8-hydroxy, 2′deoxyguanosine (8OHdG), has been used in studies to demonstrate that oxidative DNA damage is significantly elevated in the spermatozoa of patients attending infertility clinics.[34,35] Furthermore, the levels of 8OHdG expression have been shown to correlate highly with the measurement of DNA damage in spermatozoa, as measured by the TUNEL or sperm chromatin dispersion assays.[34,36] Indeed, the correlation between 8OHdG formation and DNA damage is so high that we have been forced to conclude that most DNA damage in spermatozoa is oxidatively induced. In order to understand why this would be the case we need to appreciate the particular architecture of human spermatozoa and the major points of difference with somatic cells in terms of the mechanisms regulating apoptosis.

Apoptosis is the default condition for spermatozoa. In the absence of fertilization, most spermatozoa will become senescent and default to an apoptotic state. In somatic cells, apoptosis is associated with extensive nuclear fragmentation as a consequence of nucleases released from the mitochondria (e.g., endonuclease G) or activated in the cytosol (e.g., caspase-activated DNase). However, spermatozoa are distinguished from every other cell type in biology in having a nucleus that is physically separated from the mitochondria and most of the cytoplasm. As a consequence, even when apoptosis is activated in these cells using inhibitors of PI3 kinase such as wortmannin,[37] the nucleases associated with this process remain resolutely locked within the midpiece of the cell and do not penetrate the nuclear compartment (**Figure 2**). Thus, even when apoptosis is induced in suspensions of human spermatozoa, the DNA does not become cleaved by nucleases, at least in the short-term.[37] The only products of apoptosis that can damage sperm DNA are the ROS generated by the mitochondria. Mitochondria are potent generators of ROS in spermatozoa and this activity becomes enhanced as soon as the spermatozoa default to an apoptotic state. Indeed mitochondrial ROS generation is one of the first signs that these cells have engaged the intrinsic apoptotic cascade.[37,38] It is for this reason that most of the DNA damage observed in spermatozoa is oxidative in nature.

If nucleases are ever involved, it would be at the very beginning or the very end of sperm existence. During late spermatogenesis, spermatid DNA becomes enzymatically cleaved in order to relieve the torsional stress associated with sperm chromatin compaction. Such endogenous nicks are thought to be resolved by topoisomerase before spermiation, however in pathological cases, such repair mechanisms may be deficient leading to the persistence of nicked DNA into the mature gamete.[39,40] The possibility that DNA damage in spermatozoa has its origins during spermiation is supported by the profound correlation, which has been observed between DNA fragmentation and chromatin compaction in spermatozoa as detected by chromomycin A3 fluorescence.[34,41] Viewed in this light, both DNA fragmentation and poor chromatin compaction may be regarded as independent signs of errors in spermiogenesis. An alternative explanation is that these two events are causally related. According to this 'two-step' model, errors in spermiogenesis initially lead to poor chromatin protamination and create a state of vulnerability in the spermatozoa. In the second step, spermatozoa are exposed to oxidative stress from a variety of sources including exposure to exogenous ROS as a consequence of leukocyte infiltration, or endogenous ROS triggered by entry into the intrinsic apoptotic cascade, ultimately resulting in enhanced oxidative DNA damage. Of course, this two-step hypothesis[42,43] to explain the origins of oxidative DNA damage is not necessarily exclusive of the concept that nuclease-mediated DNA nicks might persist in spermatozoa from late spermatogenesis. Nevertheless, the high correlation that has been observed between oxidative DNA damage and DNA fragmentation suggests that most of the DNA damage is occurring following spermiation as a result of enhanced vulnerability to oxidative stress.[42] The only other time that nucleases may contribute to DNA damage in the male germ line would be at the end of a spermatozoon's life when intracellular nucleases released during the perimortem as the internal structure of these cells starts to break down, or extracellular nucleases released from the male reproductive tract, may aid in the final disposal of these cells by the phagocytic armies of the immune system.[44,45]

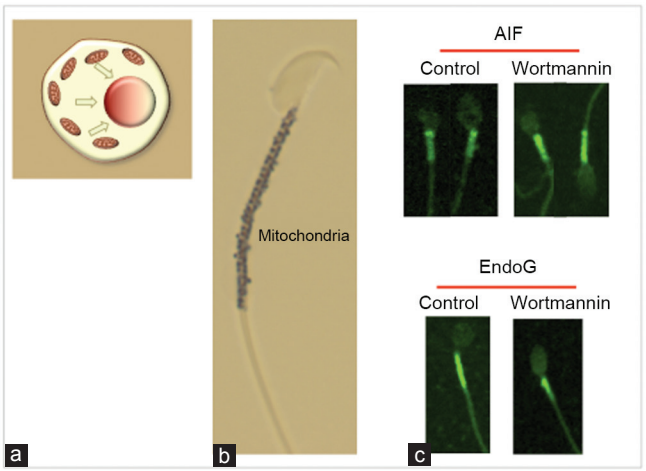

Figure 2: The unique architecture of spermatozoa influences the impact of apoptosis on DNA integrity. **(a)** Conventional somatic cells feature a centrally placed nucleus surrounded by mitochondria embedded in the cytoplasm. Under these circumstances, endonucleases activated in the cytoplasm or released from the mitochondria during apoptosis are able to move into the nucleus (arrows) and cleave the DNA. **(b)** Spermatozoa are completely different from such somatic cells because their mitochondria (stained black) and most of their cytoplasm are located in the midpiece of the cell, physically separated from the nucleus. **(c)** As a consequence of this compartmentalization key effectors of apoptosis such as apoptosis inducing factor (AIF) or Endonuclease G (Endo G) remain resolutely locked in the sperm midpiece even when apoptosis is induced by the powerful PI3 kinase inhibitor, wortmannin and cannot move into the sperm nucleus. Because of this physical limitation, most DNA damage in mature spermatozoa is induced by membrane permeant reactive oxygen species emanating from the mitochondria, rather than nucleases.

DNA REPAIR IN SPERMATOZOA

The importance of oxidative stress in the mechanisms by which sperm DNA becomes damaged is also indicated by a consideration

of the DNA repair strategies these cells are capable of employing. Incorporated into the subcellular structure of the sperm nucleus and mitochondria is an 8-oxoguanine glycosylase, known as 8-oxoguanine glycosylase 1 (OGG1).[46] When sperm DNA experiences an oxidative attack OGG1 immediately clips the 8OHdG residues out of the DNA generating an abasic site, releasing the oxidized base into the extracellular space. The next enzyme in the base excision repair (BER) pathway, APE1, then incises DNA at the phosphate groups 3' and 5' to the baseless site leaving 3'-OH and 5'-phosphate termini ready for the insertion of a new base. Spermatozoa do not possess this enzyme.[46] As a result, they carry their abasic sites into the oocyte for continuation of the repair process (**Figure 1**). For its part, the oocyte engages in a round of DNA repair immediately after fertilization and puts S-phase on hold until this activity has been completed.[47,48] If the oocyte should make a mistake during the completion of this post-fertilization repair process, it creates the potential for *de novo* mutations in the offspring which could have a profound impact on the health and well-being of the latter (**Figure 1**).

LIFESTYLE, AGE AND OXIDATIVE STRESS

Given this propensity for oxidative damage to sperm DNA and a heavy reliance on OGG1 to cleave out damaged base adducts prior to fertilization, it would not be surprising if factors that impeded OGG1 activity had a profound impact on fertility and the health of progeny. The classic inhibitor of OGG1 activity is cadmium and the latter has a long history of being associated with the etiology of male infertility.[49,50] Importantly, cadmium exposure has been shown to increase levels of DNA damage in spermatozoa[51] and positive correlations have been observed between 8OHdG levels in spermatozoa and the cadmium concentration in seminal plasma.[52] Since one of the classical sources of cadmium is cigarette smoke, it is also no surprise to learn that men who smoke heavily exhibit significantly elevated levels of oxidative DNA damage in their spermatozoa.[53] Furthermore, the impact of smoking on 8OHdG levels in human spermatozoa is significantly impacted by the presence of Ser326Cys polymorphism in the OGG1 gene.[54] Those individuals with variant Cys/Cys homozygosity for OGG1 showing higher levels of sperm 8OHdG than wildtype homozygote carriers (Ser/Ser).[53] The fact that paternal (not maternal) smoking is associated with a significant increase in the risk of childhood cancer in the offspring[55] is further testimony to the lasting clinical consequences of cigarette smoking and the power of the relationship between oxidative DNA damage in the paternal germ line and the long-term health trajectory of the offspring (**Table 1**).

If the oxidative DNA damage induced in the germ line as a consequence of smoking can impact on the incidence of cancer in the progeny, then surely any factor capable of inducing oxidative damage in spermatozoa is potentially capable of profoundly influencing the health of children. Furthermore, because there is no particular proposed order to the nature of the DNA damage or aberrant DNA repair in the oocyte, we might anticipate that the range of pathologies generated as a consequence of oxidative stress in the male germ line might be considerable. A case in point is paternal aging. It is well-recognized that as men get older they do not stop producing spermatozoa; however, the quality of their gametes exhibits a progressive age-related decline as indicated by a highly significant, age-dependent increase in sperm DNA damage.[56,57] Studies on the brown Norway rat indicate that this age-dependent increase in DNA damage in spermatozoa is associated with a concomitant down regulation of genes associated with the BER pathway and a corresponding increase in the levels of oxidative DNA damage in the spermatozoa.[58]

This relationship between paternal age and oxidative DNA damage in spermatozoa has also been indicated by recent studies on the senescence-accelerated mouse prone 8 (SAMP8). This mouse strain contains a suite of naturally occurring mutations resulting in an accelerated senescence phenotype largely mediated by oxidative stress, which is further enhanced by a mutation in the *Ogg1* gene, greatly reducing the ability of the enzyme to excise 8OHdG adducts. An analysis of the reproductive phenotype of the SAMP8 males revealed a high level of DNA damage in caudal epididymal spermatozoa as detected by the alkaline Comet assay. Furthermore, these lesions were confirmed to be oxidative in nature, as demonstrated by significant increases in 8OHdG adduct formation in the SAMP8 testicular tissue and mature spermatozoa, relative to a control strain.

If aging is associated with oxidative DNA damage to spermatozoa then we might expect to see these lesions reflected in the incidence of morbidity in the offspring of ageing fathers. In fact, we see three major kinds of paternal age-mediated pathology in the offspring; miscarriage, dominant genetic mutations and complex neurological conditions, as set out in **Table 1**. One of the first paternally-mediated pathologies to be detected was an increase in the incidence of dominant genetic diseases in children as an exponential function of their fathers' age.[59] These diseases classically include achondroplasia, Apert syndrome and multiple endocrine neoplasias.[60] The traditional explanation given for the appearance of these conditions is that they represent the

Table 1: Summary of factors that are capable of causing oxidative DNA damage in the male germ line and their consequences for the offspring

Environmental or lifestyle factor	*Sperm damage*	*Consequences for the offspring*	*References*
Smoking	Oxidative damage to sperm DNA	Increased incidence of childhood cancer	53-55
Age	Oxidative damage to sperm DNA	Increase in miscarriage Increase in dominant genetic disease Increased miscarriage Increased neurological disorders such as autism, bipolar disease, spontaneous schizophrenia and epilepsy Increased death in offspring associated with congenital malformations, injury and poisoning Increased risk of cleft palate, diaphragmatic hernia, right ventricular outflow tract obstruction and pulmonary valve stenosis	19,42,56,58-67
Infertility	Oxidative damage to sperm DNA	Unknown Possible increase in birth defects Possible increase in imprinting disorders Increased hospitalization	42,70-77
Environmental toxicants, insecticides, herbicides, heavy metals and so on	Oxidative damage to sperm DNA	Unknown	51,52,68

consequences of replication error in the germ line. As men age, their germ cells experience multiple rounds of pre-meiotic replication, and with each cellular iteration, the risk of a mutation occurring as a consequence of replication error correspondingly increases. In certain cases, such as the FGFR2 (fibroblast growth factor receptor 2) mutation associated with Apert syndrome, there does indeed appear to be a correspondence between the incidence of this mutation in spermatozoa and the appearance of the condition in children.[60] However, the underlying cause is not just replication error.[61] The mutations that cause this condition are thought to become over-represented in the sperm population as a consequence of age-dependent clonal expansion; mutant spermatogonial stem cells having a proliferative advantage over non-mutated cells. Recent studies suggest that such mutations occur in clusters within the seminiferous tubules possibly as a consequence of failures of asymmetrical division within the germ line.[60] This germ line selection model may also explain the origins of achondroplasia,[62] although in this case there does appear to be a discrepancy between the incidence of the mutation in spermatozoa and the appearance of the disease in the progeny.[63]

An alternative explanation for paternal age effects may be aberrant repair of oxidative DNA damage in the fertilized oocyte, as suggested above in the context of smoking.[64] Such a mechanism could account for the increase in miscarriage rates observed as a function of paternal age[65] and could also contribute to the etiology of a range of other complex polygenic conditions that correlate with the age of the father at the moment of conception. Thus, paternal age is also associated with an increase in the incidence of complex polygenic neurological conditions in the offspring including epilepsy, spontaneous schizophrenia, bipolar disease and autism, as well as an increased rate of death in the F1 generation associated with congenital malformations, injury and poisoning.[19] An analysis of birth defects has also revealed significant associations between paternal age with the etiology of cleft palate, diaphragmatic hernia, right ventricular outflow tract obstruction and pulmonary valve stenosis.[66] As a result of recent studies conducted on the Icelandic population, there is now powerful incontrovertible evidence that the mutational load carried by children is correlated with the age of their fathers at the moment of conception and that once this load exceeds a certain critical level, overt pathologies such as autism appear in the offspring.[67] The link between this age-dependent increase in mutational load in children and the aberrant repair of oxidative sperm DNA damage in the zygote has yet to be definitively established, however such a relationship appears probable. Furthermore, given the range of environmental and lifestyle factors that can influence oxidative stress in the germ line from pesticides to electromagnetic radiation,[68] the potential contribution of such mechanisms to the integrity of the human genome is significant (**Table 1**).

Pertinent to this debate is the global increase in the use of assisted reproductive technology (ART) to solve human infertility. In advanced western countries such as Australia, nearly 4% of newborn children are the product of assisted conception therapy.[69] Since many of these conceptions will have been triggered by male factor infertility and the latter involves a high incidence of oxidative DNA damage in the germ line, it is inevitable that conceptions are being achieved *in vitro* with severely DNA damaged spermatozoa, that could never have occurred *in vivo*.[70] One of the consequences of this trend is that we might anticipate an increase in disease incidence in children conceived using ART. The emerging data on this point is suggestive but unsubstantiated. Thus, the incidence of birth defects following ART is approximately double the background rate and there is also evidence that imprinting disorders are more frequent in children conceived *in vitro*.[71,72] Infants produced by ART are also significantly more likely to be admitted to a neonatal intensive care unit, to be hospitalized and to stay in hospital longer than their naturally conceived counterparts.[19] Recent studies have also shown an increase in the hospitalization of ART offspring in infancy and early childhood compared with spontaneously conceived children, as well as abnormal patterns of retinal vascularization and an increase in the incidence of undescended testicles in boys conceived by ICSI.[73–77]

Similarly, there are many environmental toxicants (herbicides, pesticides and so on) that will induce oxidative DNA damage in the male germ line and are therefore potential contributors to disease in the offspring.[68] Notwithstanding their possible impact, such transgenerational relationships still remain largely unexplored (**Table 1**).

DNA REPAIR IN THE GERM LINE DURING SPERMATOGENESIS

Most of the above discussion has focused on the impact of oxidative stress at the level of gamete. However, if the oxidative insult is earlier in spermatogenesis, what are the likely consequences for fertility and the health and well-being of the offspring? Under these circumstances, severe oxidative DNA damage in germ cells entering meiosis will simply precipitate an increase in apoptosis.[78] However, milder levels of oxidative stress might induce compensatory mechanisms on the part of the germ line that will favor survival of the offspring. An example of such an effect might be the impact of paternal ageing on telomere length. As discussed above, ageing is associated with oxidative stress in the germ line. One of the ways in which the germ line responds to the stresses associated with ageing is to upregulate telomerase activity and increase the length of telomeres in spermatozoa.[79] Importantly telomere length is a paternally inherited trait and so the offspring of ageing fathers also have longer telomeres.[80] Because telomere length is associated with longevity,[81] one of few positive consequences of having an older father is that he may confer upon his children the molecular basis for a long life. By contrast, if the paternal germ line has experienced an oxidative stress post-meiotically, when telomerase can no longer increase (as is typically the case in infertile patients) then telomere length in the spermatozoa will be abnormally short and the implications for the health of ART offspring, potentially serious.[82]

QUESTIONS FROM THE PANEL

Q1: Which lifestyle factors may cause oxidative stress?

A1: The factors that we know can cause oxidative stress in the male germ line are age, subfertility and smoking. However, because mitochondrial free radical generation is an early feature of apoptosis in spermatozoa, it is probable that any factor capable of compromising the vitality of male germ cells will initiate a state of oxidative stress. A list of potential factors has been compiled[68] and includes exposure to industrial pollutants such as bisphenol A, insecticides, pesticides, nonionizing electromagnetic radiation, heavy metals and a variety of small molecular mass toxicants, all of which are potentially influenced by interindividual differences in occupation and lifestyle.

Q2: What is known about oxidative stress in the mitochondria of male germ cells including spermatozoa, in response to different types of environmental chemicals (e.g., phthalates, dioxins and so on)? Is there any specificity in such responses?

A2: Any factor that causes oxidative stress in the germ line will automatically trigger mitochondrial ROS generation. It is a central feature of the intrinsic apoptotic cascade. In addition, exposure to free unsaturated fatty acids will trigger this activity by impeding the flow of electrons along the mitochondrial electron transport chain. The physiological significance of this association is indicated

by the correlation observed between the spontaneous levels of mitochondrial ROS generation by human spermatozoa and their cellular content of free arachidonic and decosahexaenoic acids.[16,17] A variety of synthetic and natural electrophiles are also capable of triggering superoxide release from the sperm mitochondria. In this context, the ability of electrophilic aldehydes (e.g., 4HNE, acrolein and malondialdehyde) generated as a consequence of lipid peroxidation to trigger mitochondrial ROS generation is particularly significant.[18] As a consequence of this pathway, any environmental factor that triggers oxidative stress in the germ line will potentiate the generation of further oxidative stress as a direct result of lipid peroxidation. Environmental factors such as dioxins are certainly capable of eliciting ROS generation from sperm mitochondria in an experimental situation.[84,85] However, whether such toxicants contribute significantly towards the oxidative stress observed in association with male infertility and sperm DNA damage is not currently understood.

Q3: Are earlier stages of spermatogenesis sensitive to ROS, and if so, does oxidative stress during fetal development play a role in the decline in sperm quality?

A3: Whether maternal exposure to reproductive toxicants during pregnancy can cause permanent changes in the germ line that might subsequently impact the fertility of the F1 generation, and the health trajectory of their offspring, is another fascinating question to which we do not yet have a definitive answer. Much will depend on the nature and intensity of the oxidative stress. In general, DNA proof reading and DNA repair in the spermatogonial stem cell population is excellent as indicated by the low risk of birth defects in the children of men with a history of cancer treatment.[86] However, the stability of the sperm epigenome may be less certain. Studies involving the maternal administration of the antiandrogenic endocrine disruptor vinclozolin, have revealed a transgenerational impact on male fertility that is mediated by a long-lasting epigenetic change in the male germ line.[87] That epigenetic changes in the germ line might be associated with impaired semen quality is therefore feasible. Furthermore, oxidative distress is known to alter the pattern of DNA methylation in spermatozoa.[88] However, whether the creation of oxidative stress in the male germ line during fetal life can subsequently influence the fertility of the male offspring, remains an interesting but unresolved possibility.

CONCLUSIONS

Oxidative stress is a major pathological mechanism responsible for both male infertility and DNA damage in the germ line. When the oxidative stress occurs in the mature gamete then 8OHdG adducts are created that are excised by OGG1; however, the remainder of the BER pathway is completed in the female germ line. Aberrant or inefficient repair on the part of the oocyte has the potential to create mutations in the offspring that will impact upon the latter's health trajectory. There is strong circumstantial evidence to support such a mechanism in that high levels of oxidative stress in spermatozoa, due to age or smoking, are known to increase the burden-of-disease subsequently carried by the offspring. Mutations in the OGG1 gene are also important contributors in this respect. Direct evidence for this causative mechanism whereby the male and female germ lines collude to increase the mutational load carried by the offspring (oxidative DNA lesions being acquired in the spermatozoa being followed by imperfect or incomplete repair in the oocyte) is currently lacking. Furthermore, we do not yet know whether the range of environmental and lifestyle factors capable of increasing oxidative DNA damage in human spermatozoa (e.g., infertility, obesity, exposure to electromagnetic radiation or environmental toxicants) have the same degree of impact on the mutation rates in the progeny. The role played by the assisted conception industry in facilitating the transfer of damaged DNA to the oocyte as a consequence of the widespread use of ICSI is also worthy of detailed scrutiny.

Finally, we do not know whether oxidative insults during fetal or prepubertal life can have a lasting impact on the genetic integrity of the germ line with implications for the health trajectory of any offspring. Studies addressing the impact of ageing on telomere length in the germ line suggest that early in spermatogenesis, germ cells are capable of exhibiting adaptive responses that may have a positive impact on offspring health. As ever, the impact of oxidative stress on reproduction is a balance of benefit and risk; quantifying the two sides of this delicate equation will be an important task for the future.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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