Chapter 2
Damage to Sperm DNA Mediated by Reactive Oxygen Species: Its Impact on Human Reproduction and the Health Trajectory of Offspring

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Abstract Disruptions to the genetic integrity of the mammalian spermatozoon play a major role in determining the subsequent developmental trajectory of the embryo. This chapter examines the causative links that connect DNA damage in human spermatozoa and the appearance of mutations in the progeny responsible for a variety of clinical conditions from autism to cancer. Integral to this discussion is an abundance of evidence indicating that human spermatozoa are vulnerable to free radical attack and the generation of oxidative DNA damage. The resolution of this damage appears to be initiated by the spermatozoa but is driven to completion by the oocyte in a round of DNA repair that follows fertilization. The persistence of unresolved oxidative DNA damage following zygote formation has the potential to create mutations/epimutations in the offspring that may have a profound impact on the health of the progeny. It is proposed that the creation of oxidative stress in the male germ line is a consequence of a wide variety of environmental/lifestyle factors that influence the health and well-being of the offspring as a consequence of mutational change.
induced by the aberrant repair of oxidative DNA damage in the zygote. Factors such as paternal age, subfertility, smoking, obesity, and exposure to a range of environmental influences, including radio-frequency electromagnetic radiation and xenobiotics, have all been implicated in this process. Identifying the contributors to oxidative stress in the germ line and resolving the mechanisms by which such stressors influence the mutational load carried by the progeny will be an important task for the future. This task is particularly pressing, given the extensive use of assisted reproductive technologies to achieve pregnancies in vitro that would have been prevented in vivo by the complex array of mechanisms that nature has put in place to ensure that only the fittest gametes participate in the generative process.

**Keywords** Reactive oxygen species (ROS) • Oxidative stress • Sperm mitochondria • DNA damage • Apoptosis • Histones • Protamines • Base excision repair

### 2.1 Introduction

The integrity of DNA in the sperm nucleus is an important determinant of semen quality since it defines not only the success of fertilization but also the normality of embryonic development and the health trajectory of the offspring. As a consequence, DNA damage in these cells is associated with the impairment of fertility, an increase in the incidence of miscarriage, and a variety of defects in the progeny ranging from neurological conditions such as autism to cancer (Evenson et al. 1999; Larson et al. 2000; Aitken and Baker 2013; Aitken et al. 2013). The backbone of the DNA helix is frequently cleaved in spermatozoa resulting in either single (SSB)- or double-strand breaks (DSB), while oxidative attack leads to the formation of base adducts particularly 8-hydroxy-2′-deoxyguanosine (8OHdG) (De Iuliis et al. 2009). The sources of this DNA damage are complex and include age, genetic disposition, lifestyle, and exposure to various external factors including ionizing radiation and a wide range of xenobiotics including chemical carcinogens (Aitken and De Iuliis 2010).

While the induction of strand breaks and progressive accumulation of base adducts can eventually give rise to mutations, there is uncertainty as to when in the reproductive process such mutagenic change occurs. In some cases, such as Apert syndrome, there is good evidence to support a model whereby the mutation causing this disease arises in spermatogonial stem cells as a function of paternal age. The consensus view of this process asserts that as men age their germ cells experience multiple rounds of premeiotic replication, and with each cellular iteration the risk of a mutation occurring as a consequence of replication error correspondingly increases. Apert syndrome involves FGFR2 (fibroblast growth factor receptor 2) mutations, which are thought to become overrepresented in the sperm population as a consequence of age-dependent clonal expansion, mutant spermatogonial stem cells having a proliferative advantage over nonmutated cells (Goriely et al. 2003). Accordingly this mutation appears in clusters within the seminiferous tubules
possibly as a consequence of failures of asymmetrical division within the germ line (Shinde et al. 2013). Such a germ line selection model may also explain the origin of other dominant genetic diseases such as achondroplasia, the incidence of which is also correlated with paternal age (Crow 2000). However, in this case, there appears to be a major discrepancy between the incidence of the causative mutation in spermatozoa and the appearance of the disease in the progeny of aging males (Hurst and Ellegren 2002). In order to explain this discrepancy, we have proposed an alternative hypothesis for the origin of such mutations whereby the latter are held to arise as a result of inefficient or aberrant repair of damaged sperm DNA within the oocyte, immediately after fertilization (Aitken et al. 2004). The first cell division following fertilization is unarguably the most important. The zygote has one opportunity to repair the DNA damage brought into the oocyte by the fertilizing spermatozoon before S-phase of the first mitotic division is initiated. Any inadequacies in this repair and replication process could have major consequences for the embryo, since any infidelities in the transmission of genetic information through the first cell division will subsequently be replicated throughout embryogenesis (Fig. 2.1).

**Fig. 2.1** Potential origins of genetic and epigenetic changes in the germ line. The “aberrant repair hypothesis” essentially posits that a variety of clinical, biological, lifestyle, and environmental factors including paternal age, obesity, gamete cryopreservation, exposure to radiation, transition metals, or a wide range of small molecular mass xenobiotics can trigger mitochondrial ROS generation and oxidative DNA damage in spermatozoa. These cells then fertilize the oocyte, possibly with the help of ART techniques such as ICSI (intracytoplasmic sperm injection), and it is then up to the oocyte to affect repair of the damaged paternal genome. Any inefficiencies or mistakes resulting from this round of postfertilization DNA repair have the potential to create mutations or epimutations that will impact upon the normal development and health trajectory of the offspring (Aitken and Krausz 2001)
Since DNA damage is such a common feature of defective spermatozoa generated by male-factor patients in an assisted reproductive technology (ART) setting (Lopes et al. 1998), it is inevitable that a significant number of in vitro conceptions are achieved using DNA-damaged spermatozoa that would have been excluded from this process in vivo. Although the use of ART and particularly ICSI (intracytoplasmic sperm injection) to achieve fertilization in cases of severe male infertility has been extremely successful in improving conception rates, such apparent therapeutic advances might have been achieved at a cost, in terms of the health and well-being of the offspring. Of critical importance to this discussion is a clear understanding of the oocyte’s competence for DNA repair. Typically, SSB are repaired by the base excision repair (BER) and nucleotide excision repair (NER) pathways, while DSB are repaired by nonhomologous end join (NHEJ) and homologous recombination (HR). The significance of these pathways in the oocyte has not been clearly articulated, but for the reasons given below, the BER pathway is probably the single most important DNA repair strategy associated with the oocyte’s central task of tackling the DNA damage brought into the zygote by the fertilizing spermatozoon.

It is the purpose of this chapter to examine the source and nature of DNA damage in human spermatozoa, to examine how such damage is repaired following fertilization, and to review how errors in this repair process impact on the health and well-being of the offspring. This discussion will begin with a consideration of the cause of DNA damage in human spermatozoa, and in this context, the spermatozoon’s susceptibility in oxidative stress is fundamental (Aitken and Clarkson 1987).

### 2.2 Oxidative Stress and Spermatozoa

#### 2.2.1 The Perpetual Cycle of ROS Generation in Spermatozoa

A major cause of DNA damage in spermatozoa is oxidative stress mediated by a variety of reactive oxygen species (ROS) including free radicals such as superoxide anion (O$_2$•$^-$), nitric oxide (NO$^-$), or the hydroxyl radical (OH$^-$) as well as powerful oxidants such as hydrogen peroxide (H$_2$O$_2$) or peroxyxinitrite (ONOO$^-$). Spermatozoa are particularly prone to oxidative stress because their antioxidant defensive capacity is limited, due to the removal of a majority of their cytoplasm during spermatogenesis and a consequential reduction in cytoplasmic antioxidants such as catalase or superoxide dismutase. Furthermore, these cells are professional generators of ROS, with a vast majority of these reactive oxygen metabolites being generated as a consequence of electron leakage from the sperm mitochondria (Koppers et al. 2008).

The vulnerability of spermatozoa to oxidative stress also reflects the abundance of substrates these cells offer up for free radical attack. Thus, the membranous constituents of spermatozoa contain high concentrations of polyunsaturated fatty acids, particularly docosahexaenoic acid, the double bonds of which are vulnerable to attack by ROS and the initiation of lipid peroxidation cascades. The hydrogen abstraction event that initiates lipid peroxidation is promoted because the carbon–hydrogen
dissociation energies are lowest at the bis-allylic methylene position, generating carbon-centered lipid radicals that then combine 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.

Further to this lipid-based vulnerability to free radical attack, we have also recently demonstrated that the lipid aldehydes generated as a result of lipid peroxidation actually trigger the generation of yet more free radicals from the sperm mitochondria in a self-perpetuating cycle (Fig. 2.2). According to this scheme, lipid aldehydes such as acrolein or 4HNE form adducts with proteins in the mitochondrial electron transport chain (ECT) that perturb the normal, controlled 4-electron reduction of oxygen to water. This results in the leakage of electrons from the ECT that affect the 1-electron reduction of nature’s universal electron acceptor, oxygen, to generate $O_2^{-•}$, which then rapidly dismutates to $H_2O_2$ under the influence of mitochondrial superoxide dismutase (Aitken et al. 2013). Intriguingly, defective spermatozoa from subfertile males contain a superabundance of superoxide dismutase, possibly reflecting the retention of excess residual cytoplasm during the terminal stages of spermiogenesis (Aitken et al. 1996; Gomez et al. 1996; Sanocka et al. 1997). The fact that correlations have also been observed between superoxide dismutase and other cytoplasmic enzymes such as glucose-6-phosphate dehydrogenase or creatine kinase supports this view (Aitken et al. 1996; Gomez et al. 1996). Normally the presence of excess superoxide dismutase would be considered an asset for any cell seeking to protect itself from oxidative stress. However, unless this enzyme is accompanied by a corresponding increase in the presence of enzymes that can scavenge $H_2O_2$ such as glutathione peroxidase or catalase (both of which are present in limited supply in human spermatozoa; Storey et al. 1998; Sanocka et al. 1997), the presence of excess superoxide dismutase simply turns a short-lived, membrane-impermeant, relatively inert free radical in the form of $O_2^{-•}$ into a long-lived, membrane-permeant reactive oxidant, $H_2O_2$, that can attack a wide variety of substrates in spermatozoa. Such attacks not only target the polyunsaturated fats that abound in the sperm plasma membrane but also the DNA in the sperm nucleus and mitochondria.

2.2.2 Primary Causes of Excess ROS Generation by Sperm Mitochondria

If oxidative stress stimulates a lipid peroxidation process characterized by the generation of aldehydes that perpetuate the generation of ROS from the sperm mitochondria, then the question arises as to what triggers oxidative stress in the first instance. In this context one of the most important activators of ROS generation by sperm mitochondria is the induction of apoptosis in response to senescence or other adverse circumstances.
2.2.2.1 The Intrinsic Apoptotic Cascade in Spermatozoa

One of the most important points to make about apoptosis in spermatozoa is that a regulated, apoptotic cell death is the default position for this cell type and is largely intrinsically induced. To the authors’ knowledge there are no extrinsic factors that will reliably and robustly trigger apoptosis in spermatozoa via a receptor-mediated mechanism. Although powerful bacterially derived factors such as lipopolysaccharide (LPS) have been claimed to trigger apoptosis in human spermatozoa (Hakimi et al. 2006),
we have been unable to consistently replicate these findings, despite many attempts. The one condition that will consistently trigger an apoptotic response in these cells is senescence, which is itself a reflection of the oxidative stress created via the formation of lipid aldehydes as a consequence of cell metabolism. If these electrophilic aldehydes are scavenged by powerful nucleophiles such as the thiol, penicillamine (Aitken et al. 2012a, b), then the survival of spermatozoa in vitro is significantly increased. The prolonged life span of mammalian spermatozoa in vivo contrasts dramatically with their limited survival in vitro and suggests that both the male and female reproductive tracts must be capable of controlling the bioavailability of such lipid aldehydes via molecular mechanisms that are still poorly unresolved.

It is the fate of a great majority of spermatozoa to experience a senescence-induced apoptotic death in either the male or female reproductive tract. Whatever the location of this death, its manner is of critical importance. Spermatozoa are terminally differentiated cells that arise long after immunological tolerance has been generated and are potentially immunogenic in both males and females. Thus, when these cells die and are phagocytosed by neutrophils or macrophages, it is important that the phagocytic process responsible for their removal is “silent” and does not trigger a pro-inflammatory, phlogistic response. Such silent phagocytosis occurs commonly in biological systems and is mediated by the phagocyte’s ability to recognize apoptotic markers such as phosphatidylserine and other “eat-me” signals on the exofacial surface of the cell being engulfed (Hochreiter-Hufford and Ravichandran 2013). In order for the massive phagocytic event that follows insemination to be truly silent, it is critical that the spermatozoa undergoing phagocytosis have undergone an apoptotic death. This apoptotic death is generally triggered by the oxidative stress that accompanies the physiological exertion needed to sustain high levels of sperm motility over prolonged periods of time.

It should also be recognized that oxidative stress is an inevitable consequence of the redox mechanisms that are needed to drive the capacitation process forward. “Capacitation” is a general term that covers a range of physiological changes that spermatozoa must undergo if they are to undergo a physiological acrosome reaction in the immediate vicinity of the oocyte. Preparation for acrosomal exocytosis involves a complex array of biochemical changes including cholesterol efflux, a quantum increase in levels of tyrosine phosphorylation, and the expression of hyperactivated movement, all of which are known to be redox regulated (de Lamirande and Gagnon 1993; Aitken et al. 1998; Brouwers et al. 2011). It is theoretically possible for a variety of ROS to be responsible for the induction of these changes associated with capacitation, but the constitutive generation of NO\(^\bullet\) by spermatozoa suggests that the powerful oxidant, peroxynitrite (ONOO\(^{-}\)), is a major product of these cells with a proven capacity to stimulate capacitation (de Lamirande and Lamothe 2009; Rodriguez et al. 2011). If fertilization does not occur, it has been proposed that the continued generation of ONOO\(^{-}\) by capacitating spermatozoa leads to a state of “over-capacitation” whereby the ROS generation that drives capacitation eventually overwhelms the limited antioxidant capacity of these cells, leading to a state of oxidative stress (Aitken 2011). The appearance of ONOO\(^{-}\)-mediated oxidative stress
leads to a loss of sperm function (Uribe et al. 2015) and the generation of lipid aldehydes that via the mechanisms described above, stimulate yet more ROS generation, ultimately precipitating a state of apoptosis characterized by rapid motility loss, mitochondrial ROS generation, caspase activation in the cytosol, annexin V binding to the cell surface, cytoplasmic vacuolization, and oxidative DNA damage (Koppers et al. 2011). During this intrinsic apoptotic cascade, it is only after the spermatozoa have become immobilized, and therefore prone to phagocytosis, that markers of apoptosis such as phosphatidylserine externalization start to appear (Koppers et al. 2011).

Sperm senescence and apoptosis is not normally induced by an external factor, but is an intrinsic process dependent on a fall in phosphoinositide 3-kinase (PI3K) activity. The latter is highly active in mammalian spermatozoa and generates the novel phosphoinositide, PtdIns(3,4,5)P3, which binds to the PH domain of kinases such as AKT1 causing the latter to translocate to the sperm surface where it becomes activated by phosphorylation under the influence of PDK-1. This pathway leads to the promotion of cell survival via a variety of mechanisms, particularly the phosphorylation and inactivation of proapoptotic proteins such as BAD (Koppers et al. 2011). If PI3K activity is suppressed with an inhibitor such as wortmannin, then AKT1 rapidly dephosphorylates, leading, in turn, to the dephosphorylation of BAD and the activation of sperm apoptosis (Koppers et al. 2011). Conversely, any pro-survival factor that stimulates PI3K activity leads to inhibition of apoptosis and prolongs the functional life span of the spermatozoa. There are, in all probability, many such pro-survival factors operating in vivo in order to achieve the prolonged survival of spermatozoa in both the male and female reproductive tracts. One of the first such pro-survival factors to be identified is prolactin (Pujianto et al. 2010). Spermatozoa possess several splice variants of the prolactin receptor, while addition of this hormone to human sperm suspensions has been found to prolong survival in association with an increase in AKT1 phosphorylation (Pujianto et al. 2010).

From the foregoing, it should be clear that oxidative stress is a feature of sperm cell biology responsible for both the induction of sperm capacitation and the eventual demise of these cells as a consequence of a senescence process that is associated with activation of the intrinsic apoptotic cascade. In vitro, sperm senescence and apoptosis are generally promoted by the lack of pro-survival factors in the culture medium. In addition, any factors that promote oxidative stress in spermatozoa have the potential to accelerate the apoptotic process. For example, just exposing spermatozoa to H2O2 will precipitate an apoptotic response (Lozano et al. 2009). Cryostorage of spermatozoa will also generate a high level of apoptosis associated with oxidative stress (Thomson et al. 2009) as will exposure to a number of free radical-generating xenobiotics (Aly 2013) including lifestyle factors such as cigarette smoke and radio-frequency electromagnetic radiation (Fraga et al. 1996; De Iuliis et al. 2009; Liu et al. 2013).

In addition it has been observed that a superabundance of polyunsaturated fatty acids will also stimulate mitochondrial ROS generation and create a state of oxidative stress in human spermatozoa; the greater the level of unsaturation, the greater the stimulatory effect (Koppers et al. 2010). Esterification of the fatty acid counters
this prooxidant 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 generating excessive levels of ROS possess abnormally high cellular contents of free polyunsaturated fatty acids, the levels of which are positively correlated with mitochondrial superoxide generation (Aitken et al. 2006). Why some men should possess spermatozoa generating particularly high levels of ROS in association with high cellular contents of polyunsaturated fatty acids is not currently known; however, such associations may reflect the impact of diet or genetic factors on reproductive function.

2.2.2.2 The Truncated Nature of Apoptosis on Spermatozoa

The reliance of spermatozoa on the intrinsic apoptotic cascade is a distinguishing characteristic of these cells that reflects their status as terminally differentiated, disposable cells. Another unique attribute of apoptosis in spermatozoa is that this process is truncated (Koppers et al. 2011). The reason for such restriction lies in the unique architecture of these cells. Unique among all cell types, spermatozoa are distinguished by the fact that all of the mitochondria and most of the cytoplasm are physically separated from the sperm nucleus (Fig. 2.3). Thus, the conventional apoptosis paradigm, involving nucleases activated in the cytoplasm or released by the mitochondria that move into a centrally located sperm nucleus in order to destroy the DNA and create the cleaved DNA ladders that characterize this process, cannot apply to this cell type. Nucleases generated in the cytoplasm or mitochondria of the sperm midpiece are physically prevented from gaining access to the nuclear DNA located in the sperm head (Koppers et al. 2011). Since chemically, DNA fragmentation can only be induced by free radicals or nucleases, the above rationale explains why most DNA damage in spermatozoa is initially oxidative in nature.

2.2.3 Oxidative Stress in Spermatozoa

High levels of oxidative DNA damage have been repeatedly observed in the spermatozoa of subfertile males (Kodama et al. 1997; Irvine et al. 2000). Oxidative damage occurs primarily at the guanine bases and causes the formation of adducts, the most common of which are 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8–oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which are commonly used as biomarkers for oxidative stress. Using a flow cytometric assay for 8OHdG, Aitken et al. (2010) demonstrated that the population of males attending an infertility clinic possessed significantly elevated levels of 8OHdG in their spermatozoa compared with an unselected control donor population. The role of ROS in causing such
increases in oxidative DNA damage in spermatozoa is suggested by the beneficial impact of antioxidants such as melatonin on the levels of 8OHdG formation observed in human spermatozoa (Bejarano et al. 2014). The source of the ROS responsible for the induction of oxidative DNA damage may be a reflection of three factors: the activation of ROS-generating phagocytic leukocytes, the generation of ROS by the spermatozoa themselves, and a lack of ROS-scavenging enzymes and small molecular mass antioxidants.

**Fig. 2.3** The unique architecture of spermatozoa has an impact on the nature of apoptosis in these cells. (a) Unlike conventional somatic cells spermatozoa are designed in such a way that the sperm nucleus is in a separate physical compartment from all of the mitochondria and most of the cytoplasm. In this image of a mouse spermatozoon, the mitochondrial gyres have been highlighted black using a histochemical stain. (b) As a result of this unusual architecture, apoptosis can be induced with, for example, wortmannin, but nucleases that become activated during this process such as endonuclease G (EndoG) or apoptosis-inducing factor (AIF) remain resolutely locked in the midpiece of the cell and cannot penetrate into the sperm nucleus. This is why most DNA damage in spermatozoa is initiated by oxidative stress rather than intracellular nucleases; the only product of apoptosis that can cross from the midpiece to the sperm head and attack the nuclear DNA is membrane-permeant ROS such as H$_2$O$_2$. (c) As a result of such factors, the release of ROS from the sperm mitochondria is highly correlated with the induction of oxidative DNA damage in these cells.
2.2.3.1 Leukocytes as a Cause of Oxidative Stress

Infiltrating phagocytic leukocytes are potentially capable of inducing oxidative stress in the male reproductive tract; however, there does not appear to be a strong direct relationship between leukocytospermia and DNA damage in spermatozoa, with data being presented to both support and refute this proposal (Saleh et al. 2002, Moskovtsev et al. 2007). The situation is complex because the degree of oxidative stress experienced by the spermatozoa will depend on the number and type leukocytes present in the ejaculate, when they entered the seminal compartment, whether the leukocytes are activated and, if so, when and how they are activated (Aitken and Baker 1995). Every human ejaculate contains a small number of leukocytes (in the order of 2–5 × 10⁴/ml) that appear to be in a free radical-generating, activated state (Aitken et al. 1995; Aitken and Baker 2013). However, the presence of such leukocytes does not seem to have a powerful effect on sperm function or create particularly high levels of oxidative stress. The reason for this may be that the leukocytes enter the seminal fluid at the level of the secondary sexual glands, particularly when infection is involved. In such instances, the first time the spermatozoa will come into contact with infiltrating leukocytes will be at the moment of ejaculation. At this juncture, the spermatozoa will be protected from extracellular ROS by the powerful antioxidant properties of seminal plasma (Jones et al. 1979; Rhemrev et al. 2000; van Overveld et al. 2000), and the impact of the leukocyte-derived ROS will be minimal. However, quite a different picture emerges when the seminal plasma is removed in the context of preparing spermatozoa for assisted conception therapy. Under these circumstances the protective impact of seminal plasma is lost, and any activated phagocytes that are present in the sperm suspension will have free rein to launch an oxidative attack on the spermatozoa, curtailing the motility of these cells and compromising their DNA integrity. As a consequence of these relationships, negative impacts have been observed between levels of leukocyte contamination in washed sperm preparation and fertilization rates in IVF programs (Krausz et al. 1994).

In order to circumvent such damage occurring during IVF treatment cycles, it is imperative that high-quality spermatozoa are isolated from the ejaculate while these cells are still protected by the antioxidants present in seminal plasma (Aitken and Clarkson 1988). For this reason, procedures such as swim-up from a washed pellet are known to be associated with high levels of oxidative stress and the iatrogenic induction of DNA damage in the spermatozoa (Twigg et al. 1998). One of the best ways to minimize oxidative DNA damage during sperm preparation for IVF is to use a simple, swim-up-from-raw-semen approach (Twigg et al. 1998). Alternative strategies for preparing spermatozoa for assisted conception therapy include the centrifugation of spermatozoa through discontinuous colloidal silicon gradients that isolate the highest quality spermatozoa on the basis of their physical density. Although such techniques are clearly successful in isolating subpopulations of spermatozoa with high levels of motility and good morphology (Aitken and Clarkson 1988), they have been found to actually increase the levels of oxidative DNA damage seen in the spermatozoa (Aitken et al. 2014). The reason for such DNA damage...
in otherwise high-quality sperm populations has been a mystery until recently, when it was revealed that commercial sperm preparation media are commonly contaminated with metals, including iron and copper, at concentrations that induce high levels of oxidative DNA damage in human spermatozoa (Aitken et al. 2014). Fortunately, the chemical chelation of such metal contaminants is sufficient to eliminate this threat to sperm DNA integrity and render such media suitable for use in an IVF context (Aitken et al. 2014).

2.2.3.2 ROS Generation by Spermatozoa

As indicated above, oxidative stress is commonly caused in spermatozoa by the excess generation of mitochondrial ROS as a consequence of their entry into the intrinsic apoptotic pathway. The induction of mitochondrial ROS generation with, for example, radio-frequency electromagnetic radiation leads to a marked increase in 8OHdG formation followed by DNA fragmentation (De Iuliis et al. 2009). There are many conditions that will trigger the entry of spermatozoa into this pathway including senescence, temperature, exposure to toxic chemicals, and aromatic amino acids (Tosic and Walton 1950; Aitken et al. 2012a, b).

2.2.3.3 Antioxidant Deficiency

Conditions that lead to a loss of antioxidant protection for the spermatozoa such as smoking, poor diet, or prolonged incubation in culture medium lacking antioxidant supplementation can also lead to oxidative stress and oxidative DNA damage in populations of spermatozoa (Fraga et al. 1996; Dalzell et al. 2003; Aitken et al. 2009).

2.2.4 Oxidative DNA Damage in Spermatozoa

In order to explain the etiology of DNA damage in the male germ line, we recently proposed a two-step hypothesis according to which two conditions have to be met in order for such damage to appear: (1) a source of ROS and (2) a state of heightened vulnerability to free radical attack on the part of the spermatozoa. In addition to the presence of oxidizable substrates such as polyunsaturated fatty acids, another key factor defining the vulnerability of spermatozoa to oxidative attack is the status of sperm chromatin. During spermiogenesis there is an extensive remodeling of nuclear chromatin in order to compact the entire haploid genome into the volume of a sperm head. This is achieved by the progressive replacement of nuclear histones with small positively charge proteins known as protamines. In eutherian mammals the proteins have evolved to contain cysteine residues that participate in the creation of intermolecular and intramolecular cross-links during epididymal transit that reinforce the compaction of the chromatin and render the DNA highly resistant to damage.
Interestingly, metatherian mammals do not possess cysteine-rich protamines, and as a result their spermatozoa do not undergo this post-testicular phase of nuclear remodeling. As a result, metatherian spermatozoa are much more vulnerable to oxidative DNA damage than their eutherian counterparts (Bennetts and Aitken 2005).

Sperm nuclear DNA is organized into doughnut-shaped toroids linked by nuclease-sensitive interlinker regions, bound to the nuclear matrix, which connect the toroids together (Ward 2010). These interlinker regions are thought to be associated with the retention of histones and to be particularly vulnerable to oxidative attack. Treating sperm with DNase 1 has been found to break apart the toroid linker regions but leaves the DNA within the toroid undamaged. Destruction of the linker regions in this manner has been shown to delay the replication of paternal DNA postfertilization and to impair embryogenesis, suggesting that the DNA contained within these particular regions of sperm chromatin encodes genes that are of critical importance in orchestrating the early stages of embryonic development (Gawecka et al. 2013). Laboratory experiments have demonstrated it is possible to remove protamines and histone-bound nucleosomes by treatment with high salt and reducing agent. This leaves only the sperm nuclear matrix with associated loop domains attached and a resulting nuclear structure that is called a “sperm nuclear halo” (Nadel et al. 1995; Kramer and Krawetz 1996). These halos are useful in giving us an idea as to which part of the chromatin is necessary for the first few cell divisions following fertilization. When sperm halos were injected into oocytes, pronucleus formation was normal and DNA replication was initiated (Shaman et al. 2007). Replication would still occur even if 50% of the DNA that was not attached to the matrix was removed. As replication will proceed as long as these nuclear matrix-associated regions are present, the interlinker domains must be where DNA replication begins. If this is the case, then oxidative damage to these non-protaminated vulnerable regions of sperm nuclear DNA would be expected to have a major impact on development. In the future, determining which particular genes are housed in these domains would be extremely helpful in understanding how oxidative DNA damage in spermatozoa can influence the developmental potential of the embryo.

Outside of these linker regions, the extent to which sperm DNA becomes protaminated varies between species and between individuals within a species. Thus, in mouse spermatozoa around 95% of histones are removed and replaced by protamines, whereas in human spermatozoa the equivalent figure is around 15% (Ward, 2010). Additional vulnerability is created in patient samples because of the inefficient protamination of sperm chromatin. Using the fluorescent probe chromomycin A3 (CMA3) to monitor the degree of sperm chromatin protamination, a very close correlation has been observed between levels of oxidative DNA damage in human spermatozoa and the efficiency of protamine deposition during spermiogenesis; the more poorly protaminated the chromatin, the higher the CMA3 signal and the greater the risk of oxidative DNA damage (De Iuliis et al. 2009).

These observations are important because they add weight to the above mentioned two-step hypothesis of oxidative DNA damage in human spermatozoa. The first step occurs during spermiogenesis and leads to poor protamination of the sperm chromatin, resulting in defective compaction of the DNA and an accompanying DNA Damage in Sperm
increased risk of oxidative attack. The second step then involves the realization of this oxidative attack as a result of the mechanisms described above (Aitken et al. 2009).

Once an oxidative attack on sperm DNA occurs, there are two pathways open to the cell. First, if the oxidative attack is severe, it will initiate the intrinsic apoptotic cascade, culminating in a loss of motility, the appearance of apoptotic markers on the sperm surface, and ultimately cell death. When spermatozoa enter the perimortem and the internal structure of these cells starts to break down, it is possible that sperm nuclear DNA fragmentation becomes accentuated via the activation of intracellular nucleases or the entry of nucleases from the extracellular fluids bathing the spermatozoa in the epididymis or vas deferens (Sotolongo et al. 2005; Boaz et al. 2008; Smith et al. 2013). The purpose of this last-gasp perimortem attack on the sperm nucleus is to facilitate the complete destruction of the sperm genome prior to the phagocytosis of these cells by the immune system. Second, if the spermatozoon is not badly damaged, the oxidative DNA damage may simply be repaired in readiness for fertilization. Counterintuitively it is this process of DNA repair that poses the greatest threat to human health because it is errors in this process that are thought to underpin the connection between oxidative DNA damage in the spermatozoa and the mutational load subsequently carried by children, with important implications for their long-term health trajectory.

2.3 Impacts of Oxidative DNA Damage in Sperm on Human Reproduction and Health

Despite the propensity for human spermatozoa to suffer from oxidative DNA damage, there is relatively little data to confirm that such damage has a major impact on male fertility. The most compelling evidence that this is the case comes from studies in which men exhibiting DNA damage or other evidence of oxidative stress in their spermatozoa have had their sperm DNA integrity or fertility improved by treatment with antioxidants. A study by Suleiman et al. (1996), for example, was distinguished by the careful selection of patients according to evidence of oxidative stress in their spermatozoa, as measured by a lipid peroxidation assay. This trial encouragingly recorded a decrease in lipid peroxidation and a resulting increase in sperm motility and fertility following treatment with vitamin E. Another study selected patients on the basis of DNA damage in their spermatozoa (measured with the TUNEL assay) and recorded a significant improvement in this criterion following treatment with an antioxidant preparation containing vitamin E as well as an accompanying increase in fertility (Greco et al. 2005). These data are certainly promising, but the data sets are too small to draw definitive conclusions about antioxidant therapy and male fertility. There is an urgent need for definitive, randomized, double-blind cross-over trials on this topic to determine the true value of antioxidant therapy in the treatment of males exhibiting high levels of oxidative DNA damage in their spermatozoa (Aitken et al. 2010). The therapeutic efficacy of male antioxidant treatment on the
maintenance of subsequent pregnancy has not yet been adequately examined, despite clear evidence linking DNA damage in spermatozoa with an increase in the incidence of miscarriage (Showell et al. 2011; Gharagozloo and Aitken 2011; Robinson et al. 2012).

In order to understand the importance of oxidative DNA damage in spermatozoa on the developmental competence of the embryo and the maintenance of pregnancy, we first need to understand how the 8OHdG lesions formed as a consequence of oxidative stress are repaired in spermatozoa and the zygote.

### 2.3.1 DNA Repair in Spermatozoa and Early Zygote

Regardless of how well defended the DNA is in sperm chromatin, some form of oxidative damage is inevitable. The base excision repair (BER) pathway plays a vital role in repairing oxidized, alkylated, and deaminated DNA bases and removing small non-helix-distorting base lesions from the genome. In most cells the BER pathway can be broken down into five major steps with each step being performed by a specific enzyme or class of enzymes. The process is highly regulated through individual protein to protein interactions and the formation of repair complexes. Incorporated into the subcellular structure of the sperm nucleus and mitochondria is the first enzyme in the BER pathway, an 8-oxoguanine glycosylase, known as OGG1. When spermatozoa experience 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 (Smith et al. 2013). The next enzyme in the base excision repair pathway, APE1, 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 (Smith et al. 2013). As a result, they carry their abasic sites into the oocyte for continuation of the repair process. Fortunately, the oocyte possesses the remaining elements of the BER pathway including APE1 and XRCC1 (T. Lord and R.J. Aitken, unpublished observations) and is able to carry the repair process through to completion. The repair of oxidative DNA lesions in spermatozoa therefore involves a high level of collision between the male and female germ lines and will be impacted not just by the levels of oxidative stress and 8OHdG formation in the spermatozoa but also the competence of the oocyte to complete the repair process initiated by the spermatozoa. The capacity of the BER pathway in the latter is clearly limited because the spermatozoa of subfertile males are known to carry significantly elevated levels of 8OHdG, which have not been excised by OGG1 (Aitken et al. 2010). It is the responsibility of the oocyte to detect these oxidized base lesions and engage in a round of DNA repair immediately after fertilization and put S-phase on hold until this activity has been completed (Gawecka et al. 2013). Interestingly, although the oocyte possesses an abundance of APE1 and XRCC1, it appears to exhibit a limited supply of OGG1 (T. Lord and R.J. Aitken, unpublished observations). As a result, we can anticipate that 8OHdG adducts will be poorly repaired by the oocyte and may persist into the first cleavage division of the embryo.
The BER pathway is not the only mechanism for dealing with oxidative DNA damage; however, it is thought to be the most important. While the mature spermatozoon has little alternative to OGG1 of the BER for repairing 8OHdG, oocytes are also known to possess alternative DNA repair pathways including nucleotide excision repair (NER) and mismatch repair (MMR) (Menez et al. 2007); however, the role of these pathways in orchestrating the oocyte’s response to oxidative DNA damage has not yet been determined.

Whatever repair mechanisms are invoked, the inefficient or aberrant repair of these 8OHdG lesions by the oocyte is known to have a negative impact on embryo quality (Meseguer et al. 2008) and has the potential to create de novo mutations or epimutations in the offspring that could have a profound impact on their health and well-being.

2.3.2 Consequences of Oxidatively Damaged DNA for Development

2.3.2.1 DNA Repair in the Zygote

Following fertilization, the integrity of the decondensed maternal and paternal chromatin is assessed by the zygote. The latter does not have transcription-coupled translation and relies on mRNA and proteins stored in the spermatozoon and oocyte for repair. There appears to be some cross-talk between the male and female pronuclei in effecting this repair, although the mechanisms are not yet understood. When irradiated mouse spermatozoa were used to fertilize oocytes, both pronuclei exhibited p53 apoptotic responses and replicated only around half of their DNA. Some zygotes did manage to progress to more advanced stages of development including implantation, but none came to term (Shimura et al. 2002; Shaman et al. 2007).

Fertilization with DNA-damaged spermatozoa may alter the expression of DNA repair genes in preimplantation embryos as early as the one-cell stage (Harrouk et al. 2000). Spermatozoa from rats subjected to cyclophosphamide resulted in zygotes with significantly higher DNA damage and higher transcripts for proteins from the nucleotide excision repair family (XPC, XPE, and PCNA), mismatch repair family (PMS1), recombination repair family (RAD50), as well as BER family members (UNG1, UNG2) (Harrouk et al. 2000). Furthermore, other studies have reported increases in gene expression from the two-cell stage onward. A gene expression profile analysis by Zeng et al. (2004) revealed that a small class of genes involved in the regulation of the cell cycle are overrepresented in two-cell mouse blastomeres compared to the zygotes. It is not currently understood why some DNA repair genes are highly expressed at particular stages of embryo development and not others. It is possible that different sources and types of DNA damage will elicit different DNA repair responses in the zygote. Regardless of which DNA repair mechanisms are involved, the time between fertilization and the first cell division is thought to be linked to the amount of DNA damage that the zygote needs to repair.
A zygote that is slow to initiate cleavage is therefore more likely to possess higher levels of DNA damage and exhibit a poorer potential for normal development (Liu et al. 2014).

The newly formed zygote is thought to be reluctant to respond to DNA damage by undergoing apoptosis (Fear and Hansen 2011) and, instead, prefers indefinite developmental arrest. It is not entirely certain if replication is arrested by the zygote to give it time to repair the DNA or because the DNA is too damaged for replication to proceed; much probably depends on the amount of DNA damage involved. Although some aspects of apoptosis, such as cytoplasmic fragmentation, will occur in the first cell cycle in mice and the second in humans, in general, the zygote will resist proapoptotic signals until it is at the 8–16 cell stage. In keeping with this proposal, Fear and Hansen (2011) found that bovine blastomeres have higher concentrations of mRNA for the antiapoptotic genes BCL2 and HSPA1A in two-cell embryos compared with their 16-cell counterparts. Thus, as the embryo develops through further cell divisions, the apoptosis pathway gradually becomes available; however, in early development it is DNA repair and developmental arrest that predominate. In a recent study of human development, for example, Burruel et al. (2014) determined that the duration of the 2nd to 3rd mitoses was most sensitive to fertilization by oxidatively damaged spermatozoa. As a result, embryos that displayed either too long or too short cytokineses at this stage of development demonstrated an increased failure to reach blastocyst stage and commit to further development.

This notion that zygotes engage in a demanding round of DNA damage recognition and repair shortly after fertilization has been exploited to develop a system whereby an embryo’s status can be noninvasively assessed by monitoring its metabolic status. With a particular focus on amino acid metabolism, Sturmey et al. (2009) found that embryos with greater viability exhibited a lower or “quieter” amino acid metabolism than those that went into arrest. It was hypothesized that this relationship exists because embryos with greater DNA damage consume more nutrients to facilitate the internal repair processes. Such differences in amino acid metabolism are significant since they could ultimately prove to be a useful marker of DNA damage when selecting embryos for transfer in an IVF context.

2.3.2.2 Environmental Factors Cause Oxidative Stress

Given the vulnerability of spermatozoa to oxidative attack and the limited capacity of the oocyte to repair oxidized DNA base adducts, there is concern that the carriage of such base lesions into the zygote may compromise the developmental competence of the embryo. Evidence in support of such concerns has largely come from an analysis of the health consequences of paternal cigarette smoking. Smoking has a long list of associated health problems including reproductive impacts. Despite the efforts made to control tobacco consumption across the world, smoking is still common, and the highest prevalence of smoking is seen among young men between the ages of 20–39, when they are likely to be fathering a child (Li et al. 2011).
Paternal, rather than maternal, smoking is associated with a significant increase in the incidence of childhood cancer in the offspring (Lee et al. 2009), implying that paternal DNA damage is the source of carcinogenic mutations. Given the heavy reliance on OGG1 to cleave out DNA base adducts prior to fertilization, any factor that impairs OGG1 has the potential to affect DNA repair in the germ line and, thence, reproductive function. The classic inhibitor of OGG1 is cadmium. Cadmium exposure has been shown to increase levels of DNA damage in spermatozoa (Oliveira et al. 2009), and a positive correlation has been found between 8OHdG in spermatozoa and cadmium concentration in seminal plasma (Xu et al. 2003). The impact of smoking on 8OHdG is also exacerbated by the presence of Ser326Cys polymorphism in the OGG1 gene (Ji et al. 2013). Individuals with variant Cys/Cys homozygosity for OGG1 had higher levels of sperm 8OHdG than wild-type homozygote carriers (Ser/Ser). In addition to the impact of genetic constitution and cadmium on OGG1, men who smoke heavily are also known to be deficient in antioxidants such as vitamin C (Fraga et al. 1996). The net result of all these factors is that the spermatozoa of men who smoke heavily possess high levels of oxidative DNA damage. These lesions do not have a dramatic impact on fertility; however, they are associated with the abovementioned increase in the incidence of cancer in the progeny (Lee et al. 2009). Although causation has not been formally established between 8OHdG lesions in spermatozoa and cancer in the children of men who smoke heavily, the circumstantial evidence is compelling.

Of course, smoking is not the only lifestyle or environmental factor that can influence levels of oxidative DNA damage in spermatozoa—another is paternal age. As males age, there is a downregulation of DNA repair genes (particularly BER), and levels of oxidative DNA damage in spermatozoa increase (Paul et al. 2011) such that a man aged over 35 will have three times the levels of DNA damage in his spermatozoa as a male below this age (Singh et al. 2003). One of the major consequences of this age-dependent increase in sperm DNA damage is a linear increase in the mutational load carried by the progeny over a paternal age range that stretches from 15 to 45 (Kong et al. 2012). As the mutational load carried by children increases, so does the incidence of diseases that are known to be associated with the age of the father at the moment of conception. Thus, paternal aging is known to be linked with many different kinds of adverse clinical conditions including elevated rates of miscarriage, increased incidences of dominant genetic disease such as achondroplasia and Apert syndrome, and an enhanced risk of neuropathology in the offspring including bipolar disease, autism, spontaneous schizophrenia, and epilepsy (Aitken et al. 2013).

Of the other factors that might cause oxidative DNA damage in the male germ line, it has become very apparent that obesity negatively influences many biological functions, and gamete health is no exception. Recent studies have demonstrated that obesity is associated with decreased semen parameters (Chavarro et al. 2010, Kort et al. 2006) and damage to germ cells in the testes. Several studies have shown a link between men with high BMI and decreased sperm DNA integrity due to oxidative
stress (Tunc et al. 2011; Bakos et al. 2011). Men who are classified as overweight or obese are frequently relying on ART and ICSI to father children. Considering the plethora of health problems associated with obesity, it would be very beneficial to understand if using ICSI to circumvent infertility passes health burdens onto successive generations. More studies, particularly from the perspective of ICSI progeny of obese fathers, are clearly required.

Spermatozoa of males seeking ART may be cryopreserved for various reasons such as the preservation of fertility as a prelude to the initiation of aggressive cancer chemotherapy. Cryopreservation has been revealed to both generate and exacerbate the extent of DNA damage in spermatozoa. The exact mechanism of cryoinjury is not yet fully understood although there appears to be a strong element of oxidative damage, as ROS generation is increased in cryopreserved sperm (Wang et al. 1997) and levels of oxidized DNA damage are similarly elevated under these conditions (Thomson et al. 2009). Importantly, the addition of antioxidants such as genistein and quercetin has been shown to ameliorate the amount of DNA damage in these cells, whereas caspase inhibitors have no effect (Thomson et al. 2009; Zribi et al. 2012). So, although cryopreservation can induce apoptosis in spermatozoa and generate a concomitant increase in caspase activity (Paasch et al. 2004; Wündrich et al. 2006), such changes are probably a consequence of oxidative stress and DNA damage, not a cause.

### 2.4 Conclusions

The integrity of sperm DNA is vital for the subsequent health trajectory of the offspring. The most common cause of DNA damage in spermatozoa is oxidative stress induced by mitochondrial ROS. The spermatozoon has a limited range of antioxidant strategies, but the tight packaging of the nuclear DNA with protamines is able to protect most of the spermatozoon’s nuclear genes from damage (Sawyer et al. 2003). However, certain regions of sperm chromatin, particularly the toroid interlinker domains, remain vulnerable (Ward 2010). After decondensation in the oocyte, these regions appear necessary for the initiation of DNA replication and successful cell division. The first cleavage division of the newly formed embryo is particularly important as any DNA changes induced at this time will continue to be replicated in all subsequent divisions. The early zygote possesses protection against the initiation of apoptosis and instead tends to put cell division on hold and during this delay attempts to repair the DNA using the BER, NER, MMR, HNEJ, and HR pathways. Thus, while most oxidative DNA damage is contributed to the zygote by the fertilizing spermatozoon, most of the responsibility for effecting adequate repair rests with the oocyte. Any mistakes made by the oocyte at this vital stage of development have the potential to result in mutations or epimutations that will influence the entire course of embryonic development.
Elucidating the mechanisms responsible for detecting and addressing DNA damage in the early zygote is important for our understanding of the developmental origins of human disease. In this context, it is clearly imperative that we identify the range of environmental and lifestyle factors responsible for inducing high levels of oxidative DNA damage in the male germ line. We already know that cigarette smoking, obesity, and advanced paternal age are associated with high levels of oxidative DNA damage in spermatozoa, and we strongly suspect that there are other factors capable of inducing such lesions, including radio-frequency electromagnetic radiation and exposure to a range of environmental toxicants and chemotherapeutic agents. Categorizing these causative factors, resolving their impact on the genetic and epigenetic profile of the progeny, and putting in place preventative measures to reduce risks to the genetic integrity of the progeny are significant tasks for gamete biologists—now and in the future. This responsibility is particularly significant given the current widespread use of ART to achieve conceptions in vitro that could not have occurred in vivo; until the genetic consequences of such trends are understood, we may be inadvertently creating a health burden for our species that future generations will have to solve.

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References

Aitken RJ, Gibb Z, Mitchell LA et al (2012a) Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucophile thiols. Biol Reprod 87(5):110. doi:10.1095/biolreprod.112.102020


