Human Reproduction, Vol.00, No.0, pp. 1–11, 2021
doi:10.1093/humrep/deab004

human reproduction

OPINION

Should we be measuring DNA damage in human spermatozoa? New light on an old question

R. John Aitken[1,*] and Hassan W. Bakos[1,2]

[1]Priority Research Centre for Reproductive Science, University of Newcastle, Callaghan, NSW 2308, Australia [2]Monash IVF Group Limited, Level 2, 1 Fennell Street, Parramatta, NSW 2151 Australia

*Correspondence address. Priority Research Centre for Reproductive Science, Life Sciences Building, University of Newcastle, Callaghan, NSW 2308, Australia. Tel: +61 2 4921 6851; E-mail john.aitken@newcastle.edu.au

Submitted on December 1, 2020; resubmitted on December 29, 2020; editorial decision on January 5, 2021

ABSTRACT: Assessments of sperm DNA damage are controversial because of perceived uncertainties over the relationship with pregnancy and the limited range of therapies available should positive results be returned. In this article, we highlight recent data supporting a chain of associations between oxidative stress in the male germ line, DNA damage in spermatozoa, defective DNA repair in the oocyte, the mutational load carried by the resulting embryo and the long-term health trajectory of the offspring. Any condition capable of generating oxidative damage in spermatozoa (age, obesity, smoking, prolonged abstinence, varicocele, chemical exposures, radiation etc.) is capable of influencing offspring health in this manner, creating a range of pathologies in the progeny including neuropsychiatric disorders and cancer. If sperm DNA damage is detected, there are several therapeutic interventions that can be introduced to improve DNA quality prior to the use of these cells in ART. We therefore argue that infertility specialists should be engaged in the diagnosis and remediation of sperm DNA damage as a matter of best practice, in order to minimize the risk of adverse health outcomes in children conceived using ART.

Key word: spermatozoa / DNA damage / oxidative stress/ offspring health / age /smoking / obesity / abstinence

Introduction

Should we measure DNA damage in spermatozoa? This year, two landmark papers have been published contemporaneously that address this difficult and complex question. These papers originated from the laboratories of Don Evenson (Evenson *et al.*, 2020) and Denny Sakkas (Vaughan *et al.*, 2020) and both analyzed the relationship that exists between paternal age and the appearance of DNA damage in human spermatozoa. They are both major works based upon the analysis of thousands of patients and both draw the same conclusion—that paternal age is a powerful risk factor for DNA damage in the male germ line. These findings echo the results of previous studies using a variety of DNA damage assessment protocols that have also reported a positive correlation between paternal age and DNA damage in human spermatozoa (Singh *et al.*, 2003; Das *et al.*, 2013; Belloc *et al.*, 2014). The appearance of these papers provides an opportunity for us to re-examine the rationale behind assessing sperm DNA damage that goes beyond the traditional 'does-it-correlate-with-fertility' debate into a consideration of the health and wellbeing of the offspring.

Fundamental to this discussion is the observation that robust linear correlations exist between paternal age, DNA damage in the spermatozoa and the mutational load subsequently carried by the offspring (Kong *et al.*, 2012; Aitken *et al.*, 2020a). Ultimately, around 75% of all *de novo* mutations in our species originate in the father's germ line and are highly correlated with paternal age (Gao *et al.*, 2019). This increase in mutational load as a consequence of paternal age is, in turn, associated with poor embryo quality (Kaarouch *et al.*, 2018), an increased risk of miscarriage (Du Fossé *et al.*, 2020) and a reduced chance of live birth (Horta *et al.*, 2019). A variety of pathologies have also been described in the offspring of ageing males including dominant genetic diseases, such as Apert syndrome and achondroplasia, a complex array of neuropsychiatric disorders including bipolar disease, spontaneous schizophrenia and autism (Janecka *et al.*, 2019; Aitken *et al.*, 2020a), an increased risk of mortality before 5 years of age (Wu *et al.*, 2020), reduced brain volume and white matter organization (Gale-Grant *et al.*, 2020), selective mutism (Koskela *et al.*, 2020), increases in cardiovascular risk factors including total cholesterol and triglycerides (Ahn and Hwang, 2019), an elevated risk of cancer, including acute lymphoblastic leukemia (Petridou *et al.*, 2018), birth defects including heart malformations and cleft pallet (Janeczko *et al.*, 2020) and, ironically, male infertility (Sharma *et al.*, 2015). Complications of pregnancy including gestational diabetes, intrauterine

growth restriction, preterm birth, miscarriage and fetal loss have also been linked to the age of the father at the moment of conception (Yatsenko and Turek, 2018; Phillips *et al.*, 2019). Given this cascade of robust correlations between paternal age, DNA damage in the spermatozoa, the mutational load carried by the offspring and pathologies affecting the latter's development, health and wellbeing, the analysis of DNA damage in human spermatozoa is considered by many to be a front-line diagnostic procedure (Aitken et al., 2013; Lewis, 2014). However, this is not a universal view.

Thus the Practice Committee of the American Society for Reproductive Medicine concluded in 2013 that 'current methods for assessing sperm DNA integrity do not reliably predict treatment outcomes and cannot be recommended routinely for clinical use' (Practice Committee of the American Society for Reproductive Medicine, 2013). Although this viewpoint is perfectly understandable we would propose, with the deepest respect, that this august body of experts has missed the point. Analyzing the chromatin status of human spermatozoa should not just be about predicting fertility, because the latter will only be compromised when levels of DNA damage are high (Evenson *et al.*, 2020). More sinister is the observation that spermatozoa showing moderate levels of DNA damage are still capable of fertilization (Aitken *et al.*, 1998) or can be forced to do so, using ICSI (Twigg *et al.*, 1998). Given the chain of associations described above between sperm DNA damage, mutational load and offspring health, it should be evident that the use of DNA-damaged spermatozoa in ART may have dire consequences for the health and wellbeing of the progeny. Thus, we would argue that the analyses of sperm DNA damage should be undertaken, as a matter of principle, reflecting our deep desire to minimize the mutational load carried by the offspring and our commitment to 'best practice' within the IVF industry (Aitken *et al.*, 2013).

When is mutational change in the male germ line induced?

In any discussion of mutational change in the male germ line, it is important to emphasize that the spermatogonial stem cell (SSC) population is extremely robust, possessing a remarkable capacity for DNA surveillance and repair even in the face of severe chemotherapeutic attack (Xavier *et al.*, 2018). Such results are in keeping with the 'disposable soma hypothesis', which posits that the genetic fidelity of germ cells will be maintained at the expense of the soma, given the former's sentinel position in safeguarding the integrity of the genome. Such results are also in keeping with clinical data, which have not revealed any evidence of increased mutational load or birth defects in the offspring of fathers previously exposed to a variety of chemotherapeutic reagents or even the fallout from atomic bombs (Kryukov *et al.*, 2016; Nørgård *et al.*, 2017; Ozasa *et al.*, 2018). Of course, there are limits to the ability of the germ line to withstand toxicant exposure (Ton *et al.*, 2018); nevertheless, we would assert that the SSC population is relatively resistant to mutational change.

The exceptions to this rule are the mutations responsible for such dominant genetic diseases as achondroplasia and Apert syndrome. These conditions are known to be closely associated with paternal age at conception (Crow, 2000) and to involve germline mutations in fibroblast growth factor receptors (FGFR)—FGFR2 in the case of Apert syndrome and FGFR3 in the case of achondroplasia. These mutations clearly do arise in the SSC population and are traditionally held to occur as a result of replication error. According to this model, the number of rounds of replication experienced by the SSC population increases linearly with age - 35 rounds of replication in a 15-year-old boy compared with 610 rounds in a 40-year-old man (Thacker, 2004). Naturally, with each round of replication there is a risk that a genetic error will occur. However, this replication error hypothesis has recently been challenged because even at the beginning of reproductive life, when gender-specific differences in germ cell replication are not apparent, the male germ line is, nevertheless, associated with a highly significant increase in the mutational load carried by the offspring (Gao *et al.*, 2019). Indeed, how could random replication error account for the incidence of a condition such as achondroplasia, which is as high as 1 in 6400 births in Denmark and around 1 in 20 000 worldwide (Foreman *et al.*, 2020)? Most *de novo* genetic mutations causing achondroplasia arise in the same nucleotide pair and result in the same glycine to arginine substitution (G380R) in the FGFR3 protein. The odds that this specific mutational change might randomly occur in a genome containing 3 billion base pairs are infinitesimally small and bear no relation at all to the observed incidence.

This enigma has been resolved by the 'selfish selection hypothesis', which posits that the FGFR mutation responsible for achondroplasia confers upon affected germ cells a selective advantage permitting their clonal expansion within the germinal epithelium and a resultant cluster (jackpot) of mutant germ cells at foci along the seminiferous tubule. Thus, a section through the testis of a 70-year-old male will reveal clusters of mutant spermatogonia each one capable of differentiating into mutant spermatozoa that will generate achondroplasia in the offspring. A similar selfish selection hypothesis has also been offered to explain the age-dependent expansion of SSC carrying the FGFR2 responsible for Apert syndrome (Goriely *et al.*, 2003; Choi *et al.*, 2008). This general concept may also apply to other dominant genetic diseases impacted by paternal age including multiple endocrine neoplasia type 2 B, where the causative mutation occurs in the *RET* proto-oncogene (Choi *et al.*, 2012) and the Noonan syndrome mutation in the *PTPN11* gene (encoding protein tyrosine phosphatase non-receptor type 11) (Liao and Mehta, 2019). However, this selfish selection hypothesis does not account for a majority of the *de novo* mutations that originate in the male germ line—some other explanation must be sought (Aitken *et al.*, 2020a).

A key to this enigma is that the high level of genetic surveillance and DNA repair that characterizes the SSC population is gradually lost as male germ cells enter the spermatogenic pathway and differentiate into spermatozoa. This lack of DNA repair capacity in the maturing gamete means that spermatozoa are particularly vulnerable to the induction of DNA damage that they cannot control. Such damage amounts to a pre-mutational change that then becomes fixed as a mutation in the few hours that elapse between fertilization and the initiation of S-phase, prior to the first mitotic division (Aitken, 2018). As we shall see (Fig. 1), the potential to generate mutations at this point in development is a reflection of the oocyte's inability to manage the very large amounts of DNA damage that the fertilizing spermatozoon is capable of introducing at the moment of fertilization. An excellent example of offspring health being impacted in this way is the glutathione peroxidase 5 knockout mouse. In this model, there is a localized

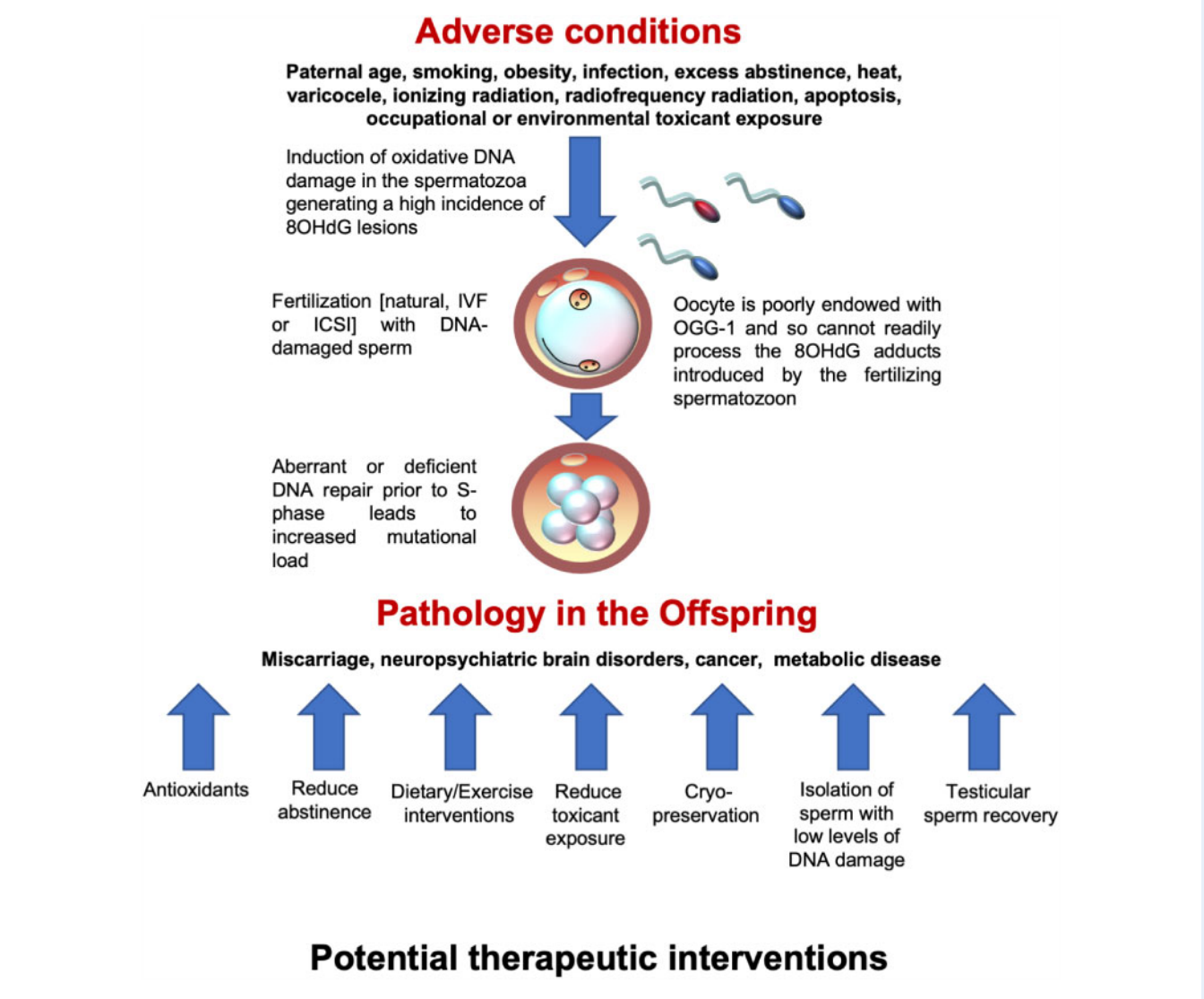

Figure 1 Chain of cause and effect between DNA damage in spermatozoa and offspring health. A variety of adverse conditions, including paternal age, smoking, obesity, infection, excess abstinence, heat, varicocele, ionizing radiation, radiofrequency radiation, apoptosis, occupational or environmental toxicant exposure are capable of inducing a state of oxidative stress in the male germ line. Spermatozoa are vulnerable to such stress resulting in the generation of oxidative DNA damage. Spermatozoa carrying oxidative DNA damage are capable of natural or assisted fertilization. Unfortunately, the oocyte is poorly equipped to deal with the highly-mutagenenic 8-hydroxy-2'-deoxyguanosine (8OHdG) DNA adducts imported by the fertilizing spermatozoon. As a result, mutations can be created as the DNA replicates in preparation for the first mitotic division. These mutations can influence the viability of pregnancy as well as the health and wellbeing of the offspring. Detecting the existence of DNA damage in spermatozoa is important because it may open up a number of therapeutic options designed to reduce the level of DNA damage present in these cells before the initiation of ART. OGG-1: 8-oxoguanine DNA glycosylase 1

loss of antioxidant protection in the epididymis and, as a result, maturing spermatozoa accumulate oxidized base lesions (particularly 8-hydroxy-2'-deoxyguanosine [8OHdG]) in their DNA. Such damage is not incompatible with fertility but does impact embryonic development, leading to increased incidences of miscarriage and birth defects in the offspring (Chabory *et al.*, 2009; Aitken, 2009). This mouse model not only shows the severe impact that sperm DNA damage can have on development, it also gives a clue as to the underlying mechanism.

How is DNA damage induced?

There are only two fundamental mechanisms by which sperm DNA can be damaged in spermatozoa: nuclease-mediated enzymatic cleavage and free radical attack. Nuclease-mediated enzymatic cleavage is known to be induced in mature spermatozoa by the presence of extracellular DNA and appears to be a defense response designed to ensure that foreign DNA does not become incorporated into germ line (Maione *et al.*, 1997; Aitken *et al.*, 2020b). This source of DNA

damage will be elevated by any mechanism that increases the amount extracellular cell-free DNA in the immediate environment of spermatozoa such as spinal cord injury (Bartolomé-Nebreda *et al.*, 2020), testicular torsion (Boettcher *et al.*, 2017) or infection (Schultz *et al.*, 2019).

In addition to the impact of extracellular DNA on mature spermatozoa, precursor germ cells might also be vulnerable to nuclease-mediated apoptosis. Thus, infertile patients destined for ICSI because of profound disruptions of spermatogenesis may show DNA strand breaks in the spermatozoa that were actually initiated by nucleases activated during apoptosis at an earlier stage of germ cell development. When this occurs at, or near, the beginning of spermatogenesis (spermatogonia to late pachytene spermatocytes) the cells are generally able to engage in a classical apoptotic response culminating in their self-deletion (Hamer *et al.*, 2003; Yoon and Rhee, 2020). However, when apoptosis is induced late in spermatogenesis, in haploid spermatids, for example, cell ablation cannot occur because these highly differentiated cells have shed the cellular machinery necessary for their effective elimination. As a result, such cells may continue to differentiate into spermatozoa while still carrying the DNA strand breaks generated by an abortive attempt at apoptosis during spermiogenesis (Sakkas and Alvarez, 2010). Alternatively, defective spermiogenesis may be associated with the defective repair of double-stranded DNA strand breaks generated physiologically during the post-meiotic processing of male germ cells (Cavé *et al.*, 2019). Disruptions at this late stage of germ development might also be expected to interfere with the careful remodeling of sperm chromatin during spermiogenesis. In such cases, the impaired packaging of chromatin within the human sperm nucleus may, in turn, create a state of vulnerability to DNA damage (De Iuliis *et al.*, 2009). However, even when such damage does occur, it does not seem to be predominantly mediated by nucleases.

This may seem a strange assertion because, in somatic cells, most DNA damage is nuclease-mediated and associated with the terminal stages of apoptosis (Arends *et al.*, 1990). However, spermatozoa are different. The archetypal somatic cell is characterized by a centrally-placed nucleus, surrounded by a sea of cytoplasm containing those harbingers of death, mitochondria. Under these circumstances, it is easy to envisage nucleases activated in the cytoplasm or released from the mitochondria moving into the dispersed chromatin of an interphase nucleus and cleaving the DNA to generate the DNA fragmentation that defines the apoptotic state. Spermatozoa are unable to replicate this behavior. Uniquely amongst all cell types, these cells partition the nucleus in a different compartment of the cell (the sperm head) than all of the mitochondria and most of the cytoplasm, which are confined to the sperm midpiece. As a result, even when an apoptotic response is initiated in these cells, any nucleases activated or released in the midpiece remain firmly locked in this cellular compartment, unable to traverse the intracellular barriers to reach the sperm head (Koppers *et al.*, 2011). Moreover, even if a nuclease, such as caspase-activated DNase, did manage to find its way into the sperm head during apoptosis, it would be confronted not with a well dispersed interphase nucleus typical of somatic cells, but with a solid mass of DNA compacted to the point of crystallization. The only product of an apoptotic sperm cell that can move readily from the midpiece to the sperm head, penetrate the densely compacted sperm chromatin and induce significant DNA damage is hydrogen peroxide (H_2O_2) generated by the sperm mitochondria (De Iuliis *et al.*, 2009; Koppers *et al.* 2011) (Fig. 1).

Oxidative attack is therefore a particularly common cause of sperm DNA damage (Kodama *et al.*,1997; De Iuliis *et al.*, 2009). In infertile men, the remodeling of sperm chromatin during spermatogenesis appears to be particularly inefficient as a result of poor protamination (Bennetts and Aitken, 2005; Ribas-Maynou *et al.*, 2020). The resultant lack of chromatin compaction is a feature of the 'two-step hypothesis' proposed by De Iuliis *et al.* (2009) to explain the etiology of DNA damage in human spermatozoa. According to this model, deficient chromatin compaction comprises the first step in the DNA-damage process, resulting in a heightened state of vulnerability on the part of the spermatozoa. The second step then involves the actual induction of DNA damage which, in light of the above discussion, is thought to be largely oxidative. The H_2O_2 generated during the apoptotic process preferentially attacks guanines, which show the lowest redox potential of the four DNA bases and are therefore the most easily oxidized, generating the base adduct, 8OHdG. As a consequence, the presence of spermatozoa expressing 8OHdG is very common in the patient population and, alarmingly, around a third of such patients are normozoospermic (Vorilhon *et al.*, 2018).

This two-step model appears relevant to the DNA damage that we see with paternal ageing, given the association observed between age-dependent sperm DNA damage and oxidative stress (Vaughan *et al.*, 2020) and the fact that the efficiency of sperm chromatin compaction decreases dramatically with age (Plastira *et al.*, 2007). The importance of age as a major contributor to oxidatively-induced DNA damage in the male germ has also been emphasized in numerous animal studies. In the brown Norway rat, for example, increased paternal age is associated with increased reactive oxygen species (ROS) production on the part of the spermatozoa, the induction of lipid peroxidation, a loss of antioxidant protection and the appearance of DNA damage in the germ line (Nguyen-Powanda and Robaire, 2020). Similarly, in another animal model of the aging process, the senescence accelerated mouse—prone 8 (SAMP8), high levels of oxidative DNA damage are observed in ageing spermatozoa relative to the control strain, SAMR1 (Smith *et al.*, 2013b). Furthermore, in the thioredoxin knockout mouse, paternal ageing is similarly associated with retrogressive changes in the spermatozoa including impaired motility, reduced chromatin compaction, increased ROS generation, lipid peroxidation and DNA damage (Smith *et al.*, 2013a). In a similar fashion, genetic deletion of the antioxidant enzyme superoxide dismutase 1, increases the levels of oxidative DNA damage detected in ageing mice (Selvaratnam and Robaire, 2016). Altogether, these animal data clearly support a model wherein ageing increases the levels of oxidative DNA damage in human spermatozoa as a result of changes in the efficiency of chromatin remodeling, increases in ROS generation and decreased levels of antioxidant protection.

Oxidative DNA damage and mutational load in the offspring

The threat imposed by the presence of 8OHdG lesions in human spermatozoa is addressed biologically by the base excision repair pathway. The first enzyme in this pathway is OGG1 (8-oxoguanine DNA

glycosylase 1). This enzyme is present in spermatozoa and serves to cleave the oxidized base out of the DNA duplex to create an abasic site (Smith *et al.*, 2013c). The next component in this pathway features an endonuclease (apurinic/apyrimidinic endodeoxyribonuclease 1 or APE-1), which cleaves the phosphodiester backbone to produce a single-nucleotide gap in the DNA with 5′-deoxyribose phosphate and 3′-hydroxyl groups at the margins. Spermatozoa do not have detectable levels of this enzyme (Smith *et al.*, 2013c). As a consequence, the base excision repair pathway stalls at this point leaving spermatozoa with unresolved abasic sites, which weakens the DNA backbone leading to fragmentation. DNA repair can, however, be resumed if the spermatozoon achieves fertilization because the oocyte is well endowed with both APE-1 and its support protein, X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1). The repair of paternal DNA damage is therefore a perfect example of male: female collaboration (Lord and Aitken, 2015).

However, even within this happy marriage, problems can arise because oocytes do not contain significant quantities of OGG-1. As a result, they find it difficult to remove any residual 8OHdG residues imported into egg by the fertilizing spermatozoon. The persistence of these highly mutagenic base lesions into the first round of embryonic DNA replication may then promote the development of an increased mutational load in the offspring (Fig. 1). These associations have been experimentally shown in genetically modified mice housing a triple knockout of genes involved in protecting DNA against the mutagenic impact of 8OHdG. The genes deleted in this model include: mutT homologue 1, which degrades 8-oxodGTP in the nucleotide pool to prevent its incorporation into DNA; OGG1, which excises 8OHdG from DNA; and mutY homologue, which removes adenine erroneously incorporated opposite 8OHdG in DNA. These triple knockout mice accumulate 8OHdG in the nuclear DNA of their gonadal cells and generate offspring carrying a high incidence of *de novo* mutations that significantly shorten their life span as a result of several pathologies including a high incidence of cancer and hydrocephalus (Ohno *et al.*, 2015). This chain of associations involving oxidative stress in the male germ line, DNA damage, the mutational load subsequently carried by the embryo and the appearance of pathology in the offspring is therefore a key feature of paternal ageing. However, we should not imagine that ageing is the only condition capable of eliciting this cascade of cause and effect.

As highlighted in Fig. 1, paternal age, smoking, obesity, infection, excess abstinence, heat, varicocele, ionizing radiation, radiofrequency radiation, apoptosis, occupational or environmental toxicant exposure and critically, male subfertility, are also capable of inducing oxidative DNA damage in spermatozoa and, thereby, enhancing the mutational load carried by the offspring and the latter's long-term health trajectory (Aitken *et al.*, 2020a). Space does not permit a detailed discussion of all these various contributors to oxidative DNA damage in the male germ line, however, we shall briefly discuss smoking, obesity and abstinence in more detail, given the significant contributions these factors make to DNA integrity in our species.

Smoking

Smoking is well known to induce oxidative DNA damage in the sperm (Fraga *et al.*, 1996; Aitken and De Iuliis, 2007). Importantly, paternal (not maternal) smoking has also been shown to induce transgenerational alterations in genome stability in the cord blood of human F1 offspring (Laubenthal *et al.*, 2012). Furthermore, smoking-induced sperm DNA damage has been implicated as a possible contributor to the etiology of childhood cancer (Chang, 2009) including acute lymphoblastic leukemia (Cao *et al.*, 2020) and non-familial sporadic heritable retinoblastoma (Kumar *et al.*, 2015). Despite the striking effects of smoking on male reproductive health, trials on the effects of smoking cessation on semen quality, as well as the transgenerational impact of such interventions, are lacking (Harlev *et al.*, 2015).

Obesity

There is clear evidence to link obesity with an increased percentage of sperm with low mitochondrial membrane potential (Fariello *et al.*, 2012; La Vignera *et al.*, 2012) and DNA fragmentation (Tunc *et al.*, 2011; Pearce *et al.*, 2019). Therefore, it has been recommended that DNA fragmentation analysis to be incorporated into semen testing, especially for obese men whose results suggest they should have normal fertility (Campbell *et al.*, 2015). Further, there is emerging evidence in animal models to suggest that the deleterious impacts of obesity are reversible with appropriate diet and exercise (Palmer *et al.*, 2012). This, coupled with evidence that diet-induced paternal obesity impairs the reproductive health of two subsequent generations (Fullston *et al*, 2012), supports the use of DNA fragmentation assays in the clinical management of such patients.

Abstinence period

The World Health Organization (WHO, 2010) recommends an abstinence period of up to 7 days prior to producing a semen profile. However, this recommendation may need to be revised downwards in light of data indicating that prolonged abstinence periods are associated with higher levels of sperm DNA damage (Bakos *et al.*, 2008; Gosálvez *et al.*, 2011; Comar *et al.* 2017). In terms of fertility, an abstinence interval of 3 days or less has been shown to be associated with higher pregnancy rates following IUI (Jurema *et al.*, 2005), whereas Scarselli *et al.* (2019) also showed that the use of a second ejaculate (produced 1 h after an initial sample) was associated with higher blastocyst euploidy rates (43.6% versus 27.5%) as well as a higher percentage of spermatozoa with mature chromatin. This study resonates with previous reports suggesting a significant improvement in sperm DNA integrity in a second sample produced 1-3 h following an initial sample (Hussein *et al.*, 2008). Similarly, it has been shown that shorter abstinence periods are associated with significant improvements in the proportion of spermatozoa showing high-velocity, progressiveness and hyperactivation in concert with a significant increase in total antioxidant capacity (Marshburn *et al.*, 2014; Alipour *et al.*, 2017).

Where in the genome is the DNA damage occurring?

If oxidative stress is such an important mediator of sperm DNA damage under a variety of circumstances, it becomes important to know

whether there are particular areas of the sperm genome that are especially vulnerable to oxidative attack. We have performed this analysis for human spermatozoa and found that, in general, susceptible regions lay outside protamine- and histone-packaged domains and are strongly correlated with interspersed repeat elements, centromeres and telomeres (Xavier *et al.*, 2019). Oxidative DNA damage was observed uniformly across the genome with two exceptions; the sex chromosomes appear to be protected from excessive DNA damage, whereas one chromosome, chromosome 15, seems to be particularly vulnerable. The area of vulnerability (approximating to 15q13-15q14) corresponds to a region of the genome to which mutations have been mapped associated with a range of neuropsychiatric disorders including spontaneous schizophrenia, bipolar disease, autism and Marfan syndrome, the incidence of which is highly correlated with paternal age (Aitken *et al.*, 2020a).

Of course, as mentioned above, ageing is just one mechanism for creating oxidative DNA damage in the sperm genome: there are many others. For example, smoking has already been mentioned as a risk factor in the creation of oxidative DNA damage in spermatozoa (Fraga *et al.*, 1996). Given the above reasoning, it would be perfectly understandable if the oxidative sperm DNA damage in males who smoke was associated with morbidity in the offspring that was targeted to chromosome 15. For example, paternal smoking is associated with a significant increase in the incidence of acute lymphoblastic leukemia in the offspring (Oldereid *et al.*, 2018) and one of the mutated genomic regions potentially associated with this condition is on chromosome 15 (Heerema *et al.*, 2002). Similarly, it would also be reasonable to expect male infertility, which is also commonly associated with oxidative DNA damage in the spermatozoa (Kodama *et al.*,1997; Vorilhon *et al.*, 2018), would be associated with diseases in the offspring where chromosome 15 is involved, particularly when ICSI is used. Reports of a significant linkage between ICSI and the incidence of autism, which can involve mutations on chromosome 15 (De Wolf *et al.*, 2013), clearly support such an assertion (Kissin *et al.*, 2015), even though the link between autism and ART is still, admittedly, contentious (Diop *et al.*, 2019). Similarly, the area of chromosome 15 which is vulnerable to oxidative attack also houses the imprinted genes responsible for Prader-Willi and Angelman syndromes. Evidence exists to suggest that both of these conditions may be elevated in the offspring of subfertile couples and is exacerbated by ART (Hattori *et al.*, 2019). Although this association between ART and imprinting disorders is not consistently observed across all datasets, the 3.44-fold increase in Prader Willi syndrome observed by Hattori *et al.* (2019) is particularly striking and could be explained by oxidative destruction of the paternal allele, allowing the maternal allele to dominate.

Clearly there is still much to discover about the links between oxidative DNA damage in spermatozoa, the application of ART and specific forms of morbidity in the offspring. As the databases needed to address this issue are growing in depth and sophistication, we can start asking questions about the most appropriate clinical response to this emerging pattern of risk.

Therapeutic options

Recognition of this link between oxidative DNA damage in spermatozoa, the mutational load subsequently carried by the embryo and the health and wellbeing of the offspring is not just an academic obsession. This knowledge can be productively used to improve the clinical management of patients by providing a rationale for modifying any lifestyle/environmental factors associated with the creation of oxidative stress in the germ line. For example, the aforementioned links between oxidative DNA damage in spermatozoa, smoking and obesity should clearly be amenable to productive interventions involving the cessation of smoking and the control of dietary intake and/or an increase in exercise, respectively (Fraga *et al.*, 1996; Pearce *et al.*, 2019). Similarly, the negative impact of prolonged abstinence on DNA integrity should encourage the deployment of short abstinence times prior to the collection of semen samples for ART. Other strategies that might be used to reduce levels of sperm DNA damage prior to ART are discussed below.

Use of testicular sperm

The use of testicular spermatozoa has been proposed as a strategy to combat elevated levels of DNA fragmentation in human spermatozoa, based on the understanding that significant DNA damage may occur as spermatozoa transit the epididymis (Esteves *et al.*, 2017). Although this strategy has been endorsed by certain bodies such as the Society of Translational Medicine (Agarwal *et al.*, 2017), recent reviews have concluded that the evidence to support this theory is weak and needs to be confirmed (Esteves *et al.*, 2018; Ambar *et al.*, 2020).

Sperm isolation for assisted reproduction

The link between sperm DNA damage and offspring health should also encourage us to look critically at the methods we are using to isolate spermatozoa for assisted conception therapy in order to select those techniques that most effectively reduce levels of DNA damage in the isolated cells (Aitken *et al.*, 2011; Aitken, 2020). The fact we still do not have a definitive answer to this question accounts for the proliferation of different sperm preparation methodologies currently used by the ART community. Swim-up from semen, swim-up from a washed pellet, glass wool columns, electrophoretic sperm isolation, density gradient centrifugation in various forms, swim down and a plethora of microfluidics systems have all been recommended in an ART context. Interestingly, it has been shown recently that the addition of ascorbic acid to the media during a swim-up procedure could reduce the percentage of ROS-positive spermatozoa and improve chromatin integrity (Raad *et al.*, 2019). Historically, vitamin E has also been shown to preserve the functional integrity of spermatozoa during their isolation (Aitken and Clarkson 1988). Systematic studies are now needed to determine the optimal combination of antioxidant supplementation and separation technology to use in the preparation of spermatozoa carrying low levels of DNA damage for assisted conception purposes.

Sperm cryopreservation

Another potential solution, particularly to DNA damage associated with age or exposure to chemotherapeutic agents, would be to consider cryostoring the spermatozoa at a young age or prior to exposure to cytotoxic reagents. The major problem with this strategy is that the methods we currently use to cryopreserve human spermatozoa induce as much oxidative DNA damage as old age (Thomson *et al.*, 2009).

Once again, the addition of antioxidants may ameliorate such damage regardless of whether conventional cryopreservation or vitrification techniques are used (Pariz *et al.*, 2019; Zhang *et al.*, 2019; Uribe *et al.*, 2020). Although, there has been no attempt to optimize the antioxidants or, indeed, the cryoprotectants that should be used in this context, recent evidence showed that commercially available cryopreservation media differ significantly in relation to their impact on sperm DNA integrity (Raad *et al.*, 2018), The entire process of human sperm cryopreservation appears to be heavily based on 20th century thinking and needs a major overhaul.

Oral antioxidants

Finally, there has been much discussion about the possible use of antioxidant treatment to reduce levels of DNA damage in human spermatozoa. Such treatments are certainly effective in animal models such as the GPx5 knockout mouse (Gharagozloo *et al.*, 2016), however appropriately designed clinical trials have not been conducted in a human context (Aitken 2020a, b). There is now an urgent need to undertake randomized, controlled, cross-over antioxidant trials, in which patient selection is based on recognized markers of oxidative stress such as 8OHdG. Moreover, the effectiveness of such treatment should be measured by the degree to which these oxidative stress markers are reduced. Furthermore, because pregnancy is influenced by a wide range of male and female factors, we recommend that it should not be used as a biomarker for assessing the efficacy of antioxidant therapy, unless female factors have been carefully eliminated and the study is appropriately powered (Aitken, 2020a, b). In this context it would also be helpful if we stopped staring at the tip of the iceberg by using the incidence of birth defects to determine whether ART is placing a genetic burden on our species. We should be using the power of next-generation sequencing technologies to conduct whole genome mutation profiling and definitively determine whether the use of DNA-damaged spermatozoa in assisted conception procedures, such as ICSI, is having a detrimental impact on the mutational load carried by future generations and whether such genetic impacts can be reversed by antioxidant treatment *in vivo*.

Conclusion

It is now clear that paternal ageing is associated with a significant increase in the levels of DNA damage, particularly oxidative DNA damage, in the spermatozoa. This deterioration in sperm DNA quality is, in turn, associated with an increase in the mutational load carried by the offspring and a variety of pathologies including neuropsychiatric disorders and cancer. Interestingly, a particular genomic hotspot for oxidative sperm DNA damage on chromosome 15 has been linked with the etiology of these conditions. This chain of associations between DNA damage in the spermatozoa, the mutational load carried by the embryo and pathological changes in the offspring suggests that any condition associated with oxidative DNA damage in human spermatozoa (age, smoking, obesity, abstinence, varicocele, chemical exposure of various kinds and, critically, male subfertility) might have similar consequences for the health and welling of future generations. We argue that patients should be screened for DNA damage, not because it correlates with the incidence of miscarriage or even live birth rate but because it provides an opportunity to intercede with a view to reducing levels of sperm DNA damage to a minimum before assisted conception procedures are used. Lifestyle modifications, reducing the abstinence period prior to ejaculation, improved sperm selection methodologies and sperm cryopreservation are all examples of strategies that we might use to reduce the incidence of such damage, should it be found. Prophylactic antioxidant therapy should also be part of sperm DNA damage management. We have shown convincingly in animal models that such treatments can bring levels of oxidative DNA damage in spermatozoa back into the normal range (Palmer *et al.*, 2012; Gharagozloo *et al.*, 2016). We now desperately need similar trials to be conducted in our own species, with the focus not so much on an enhancement in pregnancy rates but rather the resolution of oxidative DNA damage in the male gamete.

Data availability

No new data were generated or analyzed in support of this research.

Authors' roles

R.J.A. generated the original draft which was then edited and developed by H.W.B.

Funding

No specific funding was used.

Conflict of interest

R.J.A. is a consultant for Memphasys Ltd, a manufacturer of electrophoretic sperm isolation devices.

References

Agarwal A, Cho CL, Majzoub A, Esteves SC. The Society for Translational Medicine: clinical practice guidelines for sperm DNA fragmentation testing in male infertility. *Transl Androl Urol* 2017;**6**: S720–S733.

Ahn HY, Hwang IC. Paternal age at birth and metabolic risk factors in adolescents: a nationwide survey. *Public Health* 2019;**175**:1–3.

Aitken RJ. Gpx5 protects the family jewels. *J Clin Invest* 2009;**119**: 1849–1851.

Aitken RJ. Not every sperm is sacred; a perspective on male infertility. *Mol Hum Reprod* 2018;**24**:287–298.

Aitken RJ. So near yet so far away. *F&S Reports* 2020a;**1**:176.

Aitken RJ. Antioxidants on trial. *BMJ Open* 2020b;https://bmjopen.bmj.com/content/10/7/e035069

Aitken RJ. Sperm selection for ART success. *In:* J Aitken, D Mortimer, G Kovacs (eds). *Male and Sperm Factors That Maximize IVF Success*. Cambridge: Cambridge University Press, 2020, 1–14.

Aitken RJ, Bronson R, Smith TB, De Iuliis GN. The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies. *Mol Hum Reprod* 2013;**19**:475–485.

Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl* 1988;**9**:367–376.

Aitken RJ, De Iuliis GN. Value of DNA integrity assays for fertility evaluation. *Soc Reprod Fertil Suppl* 2007;**65**:81–92.

Aitken RJ, De Iuliis GN, Nixon B. The sins of our forefathers: paternal impacts on de novo mutation rate and development. *Annu Rev Genet* 2020a;**54**:1–24.

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998;**59**:1037–1046.

Aitken RJ, Hanson AR, Kuczera L. Electrophoretic sperm isolation: optimization of electrophoresis conditions and impact on oxidative stress. *Hum Reprod* 2011;**26**:1955–1964.

Aitken RJ, Whiting S, Connaughton H, Curry B, Reinheimer T, van Duin M. A novel pathway for the induction of DNA damage in human spermatozoa involving extracellular cell-free DNA. *Mutat Res* 2020b;**821**:111722.

Alipour H, Van Der Horst G, Christiansen OB, Dardmeh F, Jørgensen N, Nielsen HI, Hnida C. Improved sperm kinematics in semen samples collected after 2 h versus 4-7 days of ejaculation abstinence. *Hum Reprod* 2017;**32**:1364–1372.

Ambar RF, Agarwal A, Majzoub A, Vij S, Tadros NN, Cho C-L, Parekh N, Borges E, Glina S. The use of testicular sperm for intracytoplasmic sperm injection in patients with high sperm DNA damage: a systematic review. *World J Mens Health* 2020;**38**: https://doi.org/10.5534/wjmh.200084

Arends MJ, Morris RG, Wyllie AH. Apoptosis. The role of the endonuclease. *Am J Pathol* 1990;**136**:593–608.

Bakos HW, Thompson JG, Feil D, Lane M. Sperm DNA damage is associated with assisted reproductive technology pregnancy. *Int J Androl* 2008;**31**:518–526.

Bartolomé-Nebreda J, Vargas-Baquero E, López-Fernández C, Fernández JL, Johnston S, Gosálvez J. Free circulating DNA and DNase activity in the ejaculates of men with spinal cord injury. *Spinal Cord* 2020; doi:10.1038/s41393-020-0518-3.

Belloc S, Benkhalifa M, Cohen-Bacrie M, Dalleac A, Amar E, Zini A. Sperm deoxyribonucleic acid damage in normozoospermic men is related to age and sperm progressive motility. *Fertil Steril* 2014; **101**:1588–1593.

Bennetts LE, Aitken RJ. A comparative study of oxidative DNA damage in mammalian spermatozoa. *Mol Reprod Dev* 2005;**71**:77–87.

Boettcher M, Meier D, Jiménez-Alcázar M, Eschenburg G, Mietzsch S, Vincent D, Klinke M, Trochimiuk M, Appl B, Tiemann B *et al.* Degradation of extracellular DNA by DNase1 significantly reduces testicular damage after testicular torsion in rats. *Urology* 2017;**109**: 223.e1-223–e7.

Campbell JM, Lane M, Owens JA, Bakos HW. Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. *Reprod Biomed Online* 2015; **31**:593–604.

Cao Y, Lu J, Lu J. Paternal smoking before conception and during pregnancy is associated with an increased risk of childhood acute lymphoblastic leukemia: a systematic review and meta-analysis of 17 case-control studies. *J Pediatr Hematol Oncol* 2020;**42**:32–40.

Cavé T, Desmarais R, Lacombe-Burgoyne C, Boissonneault G. Genetic instability and chromatin remodeling in spermatids. *Genes (Basel)*. 2019;**10**:40.

Chabory E, Damon C, Lenoir A, Kauselmann G, Kern H, Zevnik B, Garrel C, Saez F, Cadet R, Henry-Berger J *et al.* Epididymis selenoindependent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J Clin Invest* 2009;**119**:2074–2085.

Chang JS. Parental smoking and childhood leukemia. *Methods Mol Biol* 2009;**472**:103–137.

Choi SK, Yoon SR, Calabrese P, Arnheim N. A germ-line-selective advantage rather than an increased mutation rate can explain some unexpectedly common human disease mutations. *Proc Natl Acad Sci U S A* 2008;**105**:10143–10148.

Choi SK, Yoon SR, Calabrese P, Arnheim N. Positive selection for new disease mutations in the human germline: evidence from the heritable cancer syndrome multiple endocrine neoplasia type 2B. *PLoS Genet* 2012;**8**:e1002420.

Comar VA, Petersen CG, Mauri AL, Mattila M, Vagnini LD, Renzi A *et al.* Influence of the abstinence period on human sperm quality: analysis of 2,458 semen samples. *JBRA Assist Reprod* 2017;**21**: 306–312.

Crow JF. The origins, patterns and implications of human spontaneous mutation. *Nat Rev Genet* 2000;**1**:40–47.

Das M, Al-Hathal N, San-Gabriel M, Phillips S, Kadoch I-J, Bissonnette F, Holzer H, Zini A. High prevalence of isolated sperm DNA damage in infertile men with advanced paternal age. *J Assist Reprod Genet* 2013;**30**:843–848.

De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B, Aitken RJ. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* 2009;**81**:517–524.

De Wolf V, Brison N, Devriendt K, Peeters H. Genetic counseling for susceptibility loci and neurodevelopmental disorders: the del15q11.2 as an example. *Am J Med Genet A* 2013;**161**: 2846–2854.

Diop H, Cabral H, Gopal D, Cui X, Stern JE, Kotelchuck M. Early autism spectrum disorders in children born to fertile, subfertile, and ART-treated women. *Matern Child Health J* 2019;**23**:1489–1499.

Du Fossé NA, van der Hoorn MP, van Lith JMM, Le Cessie S, Lashley EELO. Advanced paternal age is associated with an increased risk of spontaneous miscarriage: a systematic review and meta-analysis. *Hum Reprod Update* 2020;**26**:650–669.

Esteves SC, Roque M, Bradley CK, Garrido N. Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis. *Fertil Steril* 2017; **108**:456–467.

Esteves SC, Roque M, Garrido N. Use of testicular sperm for intracytoplasmic sperm injection in men with high sperm DNA fragmentation: a SWOT analysis. *Asian J Androl* 2018;**20**:1–8.

Evenson DP, Djira G, Kasperson K, Christianson J. Relationships between the age of 25,445 men attending infertility clinics and sperm

chromatin structure assay (SCSA®) defined sperm DNA and chromatin integrity. *Fertil Steril* 2020;**114**:311–320.

Fariello RM, Pariz JR, Spaine DM, Cedenho AP, Bertolla RP, Fraietta R. Association between obesity and alteration of sperm DNA integrity and mitochondrial activity. *BJU Int* 2012;**110**:863–867.

Foreman PK, Kessel F, Hoorn R, Bosch J, Shediac R, Landis S. Birth prevalence of achondroplasia: A systematic literature review and meta-analysis. *Am J Med Genet A* 2020;**182**:2297–2316.

Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* 1996;**351**:199–203.

Fullston T, Palmer NO, Owens JA, Mitchell M, Bakos HW, Lane M. Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* 2012;**27**:1391–1400.

Gale-Grant O, Christiaens D, Cordero-Grande L, Chew A, Falconer S, Makropoulos A, Harper N, Price AN, Hutter J, Hughes E *et al.* Parental age effects on neonatal white matter development. *Neuroimage Clin* 2020;**27**:102283.

Gao Z, Moorjani P, Sasani TA, Pedersen BS, Quinlan AR, Jorde LB, Amster G, Przeworski M. Overlooked roles of DNA damage and maternal age in generating human germline mutations. *Proc Natl Acad Sci U S A* 2019;**116**:9491–9500.

Gharagozloo P, Gutiérrez-Adán A, Champroux A, Noblanc A, Kocer A, Calle A, Pérez-Cerezales S, Pericuesta E, Polhemus A, Moazamian A *et al.* A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models. *Hum Reprod* 2016;**31**:252–262.

Gosálvez J, González-Martínez M, López-Fernández C, Fernández JL, Sánchez-Martín P. Shorter abstinence decreases sperm deoxyribonucleic acid fragmentation in ejaculate. *Fertil Steril* 2011;**96**:1083–1086.

Goriely A, McVean GA, Röjmyr M, Ingemarsson B, Wilkie AO. Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. *Science* 2003;**301**:643–646.

Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PPW, de Rooij DG. DNA double-strand breaks and gamma-H2AX signaling in the testis. *Biol Reprod* 2003;**68**:628–634.

Harlev A, Agarwal A, Gunes SO, Shetty A, Du Plessis SS. Smoking and male infertility: an evidence-based review. *World J Mens Health* 2015;**33**:143–160.

Hattori H, Hiura H, Kitamura A, Miyauchi N, Kobayashi N, Takahashi S, Okae H, Kyono K, Kagami M, Ogata T *et al.* Association of four imprinting disorders and ART. *Clin Epigenet* 2019;**11**:21.

Heerema NA, Sather HN, Sensel MG, La MKL, Hutchinson RJ, Nachman JB, Reaman GH, Lange BJ, Steinherz PG, Bostrom BC *et al.* Abnormalities of chromosome bands 15q13-15 in childhood acute lymphoblastic leukemia. *Cancer* 2002;**94**:1102–1110.

Horta F, Vollenhoven B, Healey M, Busija L, Catt S, Temple-Smith P. Male ageing is negatively associated with the chance of live birth in IVF/ICSI cycles for idiopathic infertility. *Hum Reprod* 2019;**34**:2523–2532.

Hussein TM, Elariny AF, Elabd MM, Elgarem YF, Elsawy MM. Effect of repeated sequential ejaculation on sperm DNA integrity in subfertile males with asthenozoospermia. *Andrologia* 2008;**40**:312–317.

Janecka M, Hansen SN, Modabbernia A, Browne HA, Buxbaum JD, Schendel DE, Reichenberg A, Parner ET, Grice DE. Parental age and differential estimates of risk for neuropsychiatric disorders: findings from the danish birth cohort. *J Am Acad Child Adolesc Psychiatry* 2019;**58**:618–627.

Janeczko D, Hołowczuk M, Orzeł A, Klatka B, Semczuk A. Paternal age is affected by genetic abnormalities, perinatal complications and mental health of the offspring. *Biomed Rep* 2020;**12**:83–88.

Jurema MW, Vieira AD, Bankowski B, Petrella C, Zhao Y, Wallach E, Zacur H. Effect of ejaculatory abstinence period on the pregnancy rate after intrauterine insemination. *Fertil Steril* 2005;**84**:678–681.

Kaarouch I, Bouamoud N, Madkour A, Louanjli N, Saadani B, Assou S, Aboulmaouahib S, Amzazi S, Copin H, Benkhalifa M *et al.* Paternal age: negative impact on sperm genome decays and IVF outcomes after 40 years. *Mol Reprod Dev* 2018;**85**:271–280.

Kissin DM, Zhang Y, Boulet SL, Fountain C, Bearman P, Schieve L, Yeargin-Allsopp M, Jamieson DJ. Association of assisted reproductive technology (ART) treatment and parental infertility diagnosis with autism in ART-conceived children. *Hum Reprod* 2015;**30**:454–465.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997;**68**:519–524.

Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G, Gudjonsson SA, Sigurdsson A, Jonasdottir A, Jonasdottir A *et al.* Rate of de novo mutations and the importance of father's age to disease risk. *Nature* 2012;**488**:471–475.

Koppers AJ, Mitchell LA, Wang P, Lin M, Aitken RJ. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. *Biochem J* 2011;**436**:687–698.

Koskela M, Chudal R, Luntamo T, Suominen A, Steinhausen HC, Sourander A. The impact of parental psychopathology and sociodemographic factors in selective mutism - a nationwide population-based study. *BMC Psychiatry* 2020;**20**:221.

Kryukov GV, Bielski CM, Samocha K, Fromer M, Seepo S, Gentry C, Neale B, Garraway LA, Sweeney CJ, Taplin M-E *et al.* Genetic effect of chemotherapy exposure in children of testicular cancer survivors. *Clin Cancer Res* 2016;**22**:2183–2189.

Kumar SB, Chawla B, Bisht S, Yadav RK, Dada R. Tobacco use increases oxidative DNA damage in sperm - possible etiology of childhood cancer. *Asian Pac J Cancer Prev* 2015;**16**:6967–6972.

La Vignera S, Condorelli RA, Vicari E, Calogero AE. Negative effect of increased body weight on sperm conventional and nonconventional flow cytometric sperm parameters. *J Androl* 2012;**33**:53–58.

Laubenthal J, Zlobinskaya O, Poterlowicz K, Baumgartner A, Gdula MR, Fthenou E, Keramarou M, Hepworth SJ, Kleinjans JCS, Schooten F-J *et al.* Cigarette smoke-induced transgenerational alterations in genome stability in cord blood of human F1 offspring. *FASEB J* 2012;**26**:3946–3956.

Lewis SE. Sperm DNA fragmentation and base oxidation. *Adv Exp Med Biol* 2014;**791**:103–116.

Liao J, Mehta L. Molecular Genetics of Noonan Syndrome and RASopathies. *Pediatr Endocrinol Rev* 2019;**16** (Suppl 2):435–446.

Lord T, Aitken RJ. Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Dev Biol* 2015;**406**:1–13.

Maione B, Pittoggi C, Achene L, Lorenzini R, Spadafora C. Activation of endogenous nucleases in mature sperm cells upon interaction with exogenous DNA. *DNA Cell Biol* 1997;**16**:1087–1097.

Marshburn PB, Giddings A, Causby S, Matthews ML, Usadi RS, Steuerwald N, Hurst BS. Influence of ejaculatory abstinence on seminal total antioxidant capacity and sperm membrane lipid peroxidation. *Fertil Steril* 2014;**102**:705–710.

Nørgård BM, Magnussen B, Larsen MD, Friedman S. Reassuring results on birth outcomes in children fathered by men treated with azathioprine/6-mercaptopurine within 3 months before conception: a nationwide cohort study. *Gut* 2017;**66**:1761–1766.

Nguyen-Powanda P, Robaire B. Oxidative stress and reproductive function in the aging male. *Biology (Basel)* 2020;**9**:282.

Ohno M, Sakumi K, Fukumura R, Furuichi M, Iwasaki Y, Hokama M, Ikemura T, Tsuzuki T, Gondo Y, Nakabeppu Y *et al*. 8-oxoguanine causes spontaneous de novo germline mutations in mice. *Sci Rep* 2015;**4**:4689.

Oldereid NB, Wennerholm U-B, Pinborg A, Loft A, Laivuori H, Petzold M, Romundstad LB, Söderström-Anttila V, Bergh C. The effect of paternal factors on perinatal and paediatric outcomes: a systematic review and meta-analysis. *Hum Reprod Update* 2018;**24**:320–389.

Ozasa K, Grant EJ, Kodama K. Japanese legacy cohorts: the life span study atomic bomb survivor cohort and survivors' offspring. *J Epidemiol* 2018;**28**:162–169.

Palmer NO, Bakos HW, Owens JA, Setchell BP, Lane M. Diet and exercise in an obese mouse fed a high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol Endocrinol Metab* 2012;**302**:E768–E780.

Pariz JR, Ranéa C, Monteiro RAC, Evenson DP, Drevet JR, Hallak J. Melatonin and caffeine supplementation used, respectively, as protective and stimulating agents in the cryopreservation of human sperm improves survival, viability, and motility after thawing compared to traditional TEST-Yolk buffer. *Oxid Med Cell Longev* 2019;**2019**:1–10.

Pearce KL, Hill A, Tremellen KP. Obesity related metabolic endotoxemia is associated with oxidative stress and impaired sperm DNA integrity. *Basic Clin Androl* 2019;**29**: doi:10.1186/s12610-019-0087-5.

Petridou ET, Georgakis MK, Erdmann F, Ma X, Heck JE, Auvinen A, Mueller BA, Spector LG, Roman E, Metayer C *et al*. Advanced parental age as risk factor for childhood acute lymphoblastic leukemia: results from studies of the Childhood Leukemia International Consortium. *Eur J Epidemiol* 2018;**33**:965–976.

Phillips N, Taylor L, Bachmann G. Maternal, infant and childhood risks associated with advanced paternal age: the need for comprehensive counseling for men. *Maturitas* 2019;**125**:81–84.

Plastira K, Msaouel P, Angelopoulou R, Zanioti K, Plastiras A, Pothos A, Bolaris S, Paparisteidis N, Mantas D. The effects of age on DNA fragmentation, chromatin packaging and conventional semen parameters in spermatozoa of oligoasthenoteratozoospermic patients. *J Assist Reprod Genet* 2007;**24**:437–443.

Practice Committee of the American Society for Reproductive Medicine. The clinical utility of sperm DNA integrity testing: a guideline. *Fertil Steril* 2013;**99**:673–677.

Raad G, Lteif L, Lahoud R, Azoury J, Azoury J, Tanios J, Hazzouri M, Azoury J. Cryopreservation media differentially affect sperm motility, morphology and DNA integrity. *Andrology* 2018;**6**:836–845.

Raad G, Mansour J, Ibrahim R, Azoury J, Azoury J, Mourad Y, Fakih C, Azoury J. What are the effects of vitamin C on sperm functional properties during direct swim-up procedure? *Zygote* 2019;**27**:69–77.

Ribas-Maynou J, Abad C, García-Segura S, Oliver-Bonet M, Prada E, Amengual MJ, Navarro J, Benet J. Sperm chromatin condensation and single- and double-stranded DNA damage as important parameters to define male factor related recurrent miscarriage. *Mol Reprod Dev* 2020;**87**:1126–1132.

Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 2010;**93**:1027–1036.

Scarselli F, Cursio E, Muzzì S, Casciani V, Ruberti A, Gatti S, Greco P, Varricchio MT, Minasi MG, Greco E *et al*. How 1 h of abstinence improves sperm quality and increases embryo euploidy rate after PGT-A: a study on 106 sibling biopsied blastocysts. *J Assist Reprod Genet* 2019;**36**:1591–1597.

Schulz M, Zambrano F, Schuppe H-C, Wagenlehner F, Taubert A, Ulrich G, Sánchez R, Hermosilla C. Determination of leucocyte extracellular traps (ETs) in seminal fluid (ex vivo) in infertile patients-A pilot study. *Andrologia* 2019;**51**:e13356.

Selvaratnam JS, Robaire B. Effects of aging and oxidative stress on spermatozoa of superoxide-dismutase 1- and catalase-null mice. *Biol Reprod* 2016;**95**:60.

Sharma R, Agarwal A, Rohra VK, Assidi M, Abu-Elmagd M, Turki RF. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reprod Biol Endocrinol* 2015;**13**:35.

Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. *Fertil Steril* 2003;**80**:1420–1430.

Smith TB, Baker MA, Connaughton HS, Habenicht U, Aitken RJ. Functional deletion of Txndc2 and Txndc3 increases the susceptibility of spermatozoa to age-related oxidative stress. *Free Radic Biol Med* 2013a;**65**:872–881.

Smith TB, De Iuliis GN, Lord T, Aitken RJ. The senescence-accelerated mouse prone 8 as a model for oxidative stress and impaired DNA repair in the male germ line. *Reproduction* 2013b;**146**:253–262.

Smith TB, Dun MD, Smith ND, Curry BJ, Connaughton HS, Aitken RJ. The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *J Cell Sci* 2013c;**126**:1488–1497.

Thacker PD. Biological clock ticks for men, too: genetic defects linked to sperm of older fathers. *JAMA* 2004;**291**:1683–1685.

Ton ND, Nakagawa H, Ha NH, Duong NT, Nhung VP, Hien LTT, Hue HTT, Hoang NH, Wong JH, Nakano K *et al*. Whole genome sequencing and mutation rate analysis of trios with paternal dioxin exposure. *Hum Mutat* 2018;**39**:1384–1392.

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* 2009;**24**:2061–2070.

Tunc O, Bakos HW, Tremellen K. Impact of body mass index on seminal oxidative stress. *Andrologia* 2011;**43**:121–128.

Twigg JP, Irvine DS, Aitken RJ. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum Reprod* 1998;**13**:1864–1871.

Uribe P, Cárcamo C, Navarro E, Sepúlveda J, Zambrano F, Schulz M, Sánchez R. Protective effect of the superoxide dismutase mimetic MnTBAP during sperm vitrification process. *Andrologia* 2020;**52**: e13665.

Vaughan DA, Tirado E, Garcia D, Datta V, Sakkas D. DNA fragmentation of sperm: a radical examination of the contribution of oxidative stress and age in 16 945 semen samples. *Hum Reprod* 2020; **35**:2188–2196.

Vorilhon S, Brugnon F, Kocer A, Dollet S, Bourgne C, Berger M, Janny L, Pereira B, Aitken RJ, Moazamian A *et al*. Accuracy of human sperm DNA oxidation quantification and threshold determination using an 8-OHdG immuno-detection assay. *Hum Reprod* 2018;**33**:553–562.

World Health Organization*WHO Laboratory Manual for the Examination and Processing of Human Semen*, **5**th edn. Geneva: World Health Organisation, 2010.

Wu H, Zhao M, Liang Y, Xi B. Association between paternal age and offspring's under-5 mortality: data from 159 surveys in 67 low- to middle-income countries. *J Paediatr Child Health* 2020;**56**: 1577–1583.

Xavier MJ, Mitchell LA, McEwan KE, Scott RJ, Aitken RJ. Genomic integrity in the male germ line: evidence in support of the disposable soma hypothesis. *Reproduction* 2018;**156**:269–282.

Xavier MJ, Nixon B, Roman SD, Scott RJ, Drevet JR, Aitken RJ. Paternal impacts on development: identification of genomic regions vulnerable to oxidative DNA damage in human spermatozoa. *Hum Reprod* 2019;**34**:1876–1890.

Yatsenko AN, Turek PJ. Reproductive genetics and the aging male. *J Assist Reprod Genet* 2018;**35**:933–941.

Yoon J, Rhee K. Whole-body heat exposure causes developmental stage-specific apoptosis of male germ cells. *Mol Reprod Dev* 2020; **87**:680–691.

Zhang X, Lu X, Li J, Xia Q, Gao J, Wu B. Mito-Tempo alleviates cryodamage by regulating intracellular oxidative metabolism in spermatozoa from asthenozoospermic patients. *Cryobiology* 2019; **91**:18–22.