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REVIEW

Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility

C Wright [a,*], S Milne [b], H Leeson [b]

[a] *MEDIVICE Independent Research, 10 Eden Crescent, Rathfarnham, Dublin 16, Ireland;* [b] *Positive Nutrition, 'Orwellness', 10 Orwell Road, Rathgar, Dublin 6, Ireland*

[*] Corresponding author. *E-mail addresses:* ciara.wright@medivice.ie (C Wright), heather@positivenutrition.ie (H Leeson).

Ciara Wright holds a PhD in molecular biology, obtained in 2007 at the Institute of Immunology at the National University of Ireland, Maynooth. She has worked at the postdoctoral level in cellular and molecular biology. She is currently working toward completion of a diploma in Nutritional Therapy at the Institute of Health Sciences, Dublin. Her area of interest is combining reproductive medicine with nutrition and a holistic approach to the treatment of infertility. She is an independent researcher, working with Positive Nutrition, a clinic in Dublin specializing in women's health and fertility.

Abstract DNA fragmentation is an important factor in the aetiology of male infertility. However, it is still underevaluated and its inclusion in routine semen analysis is debated. DNA fragmentation has been shown to be a robust indicator of fertility potential, more so than conventional semen parameters. Men with high DNA fragmentation levels have significantly lower odds of conceiving, naturally or through procedures such as intrauterine insemination and IVF. Couples may be counselled to proceed directly to intracytoplasmic sperm injection as it is more successful in this group, avoiding costly procedures, recurrent failures or pregnancy losses; however, this treatment is not without limitations or risks. Ideally DNA fragmentation should be minimized where possible. Oxidative stress is the major cause of DNA fragmentation in spermatozoa. Endogenous and exogenous factors that contribute to oxidative stress are discussed, and in many cases are shown to be easily modifiable. Antioxidants play a protective role, although a delicate balance of reduction and oxidation is required for essential functions, including fertilization. Reducing oxidative stress may improve a couple's chances of conception either naturally or via assisted reproduction. Sources of oxidative stress therefore should be thoroughly examined in men with high levels of DNA fragmentation and modified where possible.

KEYWORDS: antioxidants, DNA fragmentation, ICSI, male infertility, oxidative stress, ROS

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Introduction

DNA fragmentation is now considered an important factor in the aetiology of male infertility (Erenpreiss et al., 2008; Venkatesh et al., 2011; Zhang et al., 2008). However, DNA fragmentation is not routinely assessed in semen analysis according to World Health Organization (WHO) guidelines (WHO, 2010) and approximately 15–30% of couples are 'diagnosed' with unexplained infertility after a routine analysis (Practice Committee of the American Society for Reproductive Medicine, 2006b; Ray et al., 2012). DNA damage may be present in men with both abnormal and normal semen parameters (Erenpreiss et al., 2008; Oleszczuk et al., 2013) and routine semen parameters are not robustly predictive of infertility or outcome of assisted reproduction treatment (Guzick et al., 2001; Virro et al., 2004). It is still debated whether DNA fragmentation should become part of the routine analysis in fertility investigation (Practice Committee of the American Society for Reproductive Medicine, 2006a, 2008, 2013), although many research groups are greatly in favour of promoting this test (Abu et al., 2012; Bungum, 2012; Giwercman et al., 2010; Lewis et al., 2013; Omran et al., 2013).

The level of DNA fragmentation correlates negatively with pregnancy and delivery in both natural and assisted conceptions, although not after intracytoplasmic sperm injection (ICSI), as will be discussed (Evenson and Wixon, 2006; Zhang et al., 2008; Zini, 2011). It is also strongly associated with recurrent spontaneous abortion (Gil-Villa et al., 2009, 2010; Kennedy et al., 2011; Zini, 2011; Kumar et al., 2012). Using DNA fragmentation analysis routinely may allow couples to avoid costly assisted reproduction treatments, repeated failures or recurrent pregnancy losses by proceeding directly to ICSI. In addition, the source of DNA damage may be assessed and relevant treatment may increase the likelihood of spontaneous conception or successful pregnancy using assisted reproduction technology and/or ICSI. The most common cause of DNA fragmentation in spermatozoa is reactive oxygen species (ROS) and oxidative stress (Aitken and De Iuliis, 2010). This may be an area where treatment is warranted in subfertility or ahead of proceeding to assisted reproduction treatment. This area is the focus of this review.

Functions of ROS in spermatozoa

'Reactive oxygen species' is a collective common term that includes highly oxidative radicals such as hydroxyl radicals ($OH^{\cdot}$) and nonradical species such as the superoxide anion (O_2^{-}) or hydrogen peroxide (H_2O_2). The term can also include reactive nitrogen species (Doshi et al., 2012), and both species are normal byproducts of metabolism (Valko et al., 2007). Low concentrations of ROS are required for many cellular processes while overproduction is controlled and/or ameliorated by antioxidants. In spermatozoa, ROS are required for a number of specific and essential functions, which explains why they produce ROS themselves. The principal type of ROS produced in spermatozoa is O_2^{-}, which spontaneously generates H_2O_2 (Aitken and Clarkson, 1987; Alvarez et al., 1987). Due to their short half-life, these ROS are relatively harmless in spermatozoa under normal circumstances and antioxidant mechanisms help to maintain the key balance that is required for ROS-related functions (Sharma and Agarwal, 1996).

ROS, including H_2O_2 (Rivlin et al., 2004), O_2^{-} and nitrogen oxide (NO), play an important role in capacitation (de Lamirande and Lamothe, 2009; Doshi et al., 2012). Capacitation is a term that describes a complex series of events that occurs post ejaculation in the female genital tract to allow spermatozoa to reach the oocyte, bind to the zona pellucida and fertilize the egg (de Lamirande et al., 1997). ROS induce cyclic adenosine monophosphate (cAMP) in spermatozoa, inhibit tyrosine phosphatase and drive essential tyrosine phosphorylation (Leclerc et al., 1996, 1997; O'Flaherty et al., 2006). The localization of tyrosine phosphorylation to the flagellum is linked with hyperactivation (Leclerc et al., 1997; Nassar et al., 1999). Hyperactivation describes the change in motility of spermatozoa once in the female genital tract that allows chemotaxis towards, and interaction with, the oocyte (de Lamirande et al., 1997). Tyrosine phosphorylation also correlates with binding of the spermatozoon to the zona pellucida (Liu et al., 2006; Urner et al., 2001) and is necessary for the acrosome reaction (Dona et al., 2011b; Varano et al., 2008).

In vitro experiments have demonstrated the fine balance of ROS and antioxidants that are required for capacitation and ultimately fertilization. H_2O_2 can stimulate capacitation and tyrosine phosphorylation *in vitro*, while the antioxidant enzyme catalase (CAT) prevents this (Aitken et al., 1995). Another study showed more specifically that, while low concentrations of H_2O_2 promoted capacitation, elevated concentrations acted to reduce hyperactivation, zona pellucida binding and the acrosome reaction (Oehninger et al., 1995). Specific physiological concentrations of NO have also been shown to drive hyperactivation, capacitation and zona pellucida binding, while excess concentrations have inhibitory effects (Doshi et al., 2012). ROS are also required for chemotaxis, but with prolonged exposure chemotaxis is inhibited (Sanchez et al., 2010). Antioxidants are generally used to inhibit capacitation reactions *in vitro*: addition of superoxide dismutase (SOD) can inhibit capacitation (de Lamirande et al., 1998) and reverse hyperactivation (de Lamirande and Gagnon, 1993), while addition of ascorbic acid can prevent tyrosine phosphorylation and the acrosome reaction (Dona et al., 2011b).

In addition, the correct balance of ROS and antioxidants is required for chromatin compaction in maturing spermatozoa during epididymal transit. During spermiogenesis (the final stage of spermatogenesis), histones are replaced by transitional proteins and then protamines (P1 and P2), which tightly compact chromatin in toroid structures (Gonzalez-Marin et al., 2012). Further compaction occurs in the epididymis during maturation when disulphide bonds are created by the oxidation of thiol groups. Sperm-specific nuclear glutathione peroxidise (nGPX4) utilizes thiol donors for the reduction of ROS (Conrad et al., 2005; Pfeifer et al., 2001). GPX5, bound near the acrosomal membrane, may also play an oxidative role in forming disulphide bonds, whereas free or otherwise membrane-bound GPX5 has an antioxidant role, protecting spermatozoa from oxidative damage in this environment by tightly controlling the concentration of H_2O_2 (Drevet, 2006). Concentrations of these GPX proteins then decrease as spermatozoa travel

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from the caput to the cauda region of the epididymis during maturation (Seligman et al., 2005; Weir and Robaire, 2007). Incorrect compaction of chromatin by the time spermatozoa reach the cauda region is associated with abnormal morphology and infertility (Conrad et al., 2005; Hammadeh et al., 2001; Molina et al., 2001).

Oxidative stress causes DNA damage in spermatozoa

Oxidative stress occurs when the concentration of ROS becomes too high and/or antioxidant defences become overwhelmed and is linked with many disease states (Valko et al., 2007). Increased concentrations of seminal ROS have been detected in infertile men (Aktan et al., 2013; Chen et al., 2012; Moustafa et al., 2004). Highly oxidative ROS cause damage to cell components, particularly lipids, proteins and DNA (Valko et al., 2006). Two major effects of oxidative stress impacting fertility are lipid peroxidation and DNA damage.

Docosahexapentanoic acid is the predominant fatty acid in immature spermatozoa (Lenzi et al., 2000) although the percentage is reduced during epididymal maturation (Ollero et al., 2000, 2001). Due to the high proportion of this polyunsaturated acid, maturing sperm membranes are particularly vulnerable to lipid peroxidation. Lipid peroxidation occurs when the double bonds of an unsaturated fatty acid (docosahexapentanoic acid has six) are attacked by a free radical to create a lipid peroxide radical. This reaction is self-propagating by reacting with neighbouring fatty acid molecules, causing marked damage to lipid membranes and affecting membrane fluidity (Mylonas and Kouretas, 1999). Lipid peroxidation is commonly measured by quantification of the peroxidation product malondialdehyde. As shown in numerous studies, increased concentrations of malondialdehyde correlate with maturation arrest and decreased spermatozoa concentration and morphology and, most notably, motility, due to alterations in the membrane (Atig et al., 2012; Benedetti et al., 2012; Hsieh et al., 2006; Kao et al., 2008; Koksal et al., 2003). Measurements of malondialdehyde also positively correlate with DNA damage in spermatozoa, indicating that oxidative stress may link these two (Aktan et al., 2013; Atig et al., 2013; Shamsi et al., 2009). Products of lipid peroxidation are both mutagenic and genotoxic to DNA (Luczaj and Skrzydlewska, 2003).

Oxidative stress is considered to be the major cause of DNA damage in spermatozoa (Aitken and De Iuliis, 2010). Decreased levels of individual and total antioxidant capacity and high concentrations of seminal ROS have been detected in men with elevated DNA damage in numerous studies (Aktan et al., 2013; Atig et al., 2012; Khosravi et al., 2012; Mahfouz et al., 2010; Shamsi et al., 2012). 8-Hydroxy-2-deoxyguanosine (8-OHdG), an oxidized guanine base adduct formed when DNA is damaged by the $OH^{\cdot}$ radical, can be used to measure DNA oxidative damage. Increased 8-OHdG concentration correlates markedly and significantly ($r = 0.756$, $P < 0.001$) with DNA fragmentation and strand breaks (Aitken et al., 2010; De Iuliis et al., 2009b).

ROS can damage DNA directly by generation of oxidized DNA adducts (e.g. 8-OHdG, 1,N6-ethenoadenosine, 1,N6-ethenoguanosine), leading to abasic sites that destabilize the DNA structure and cause subsequent single-strand breaks (Badouard et al., 2008). NO can induce abasic sites also through the processes of nitration or deamination (Doshi et al., 2012). It was suggested that activation of caspases by ROS also caused indirect DNA damage via endonuclease activation (Sakkas and Alvarez, 2010). However, it has also been proposed that endonucleases are physically separate from activated caspases in spermatozoa (Aitken and Koppers, 2011). ROS stimulation of caspases may induce further free radical release from mitochondria during the initiation of apoptosis, further propagating damage (Aitken and Koppers, 2011; Sakkas and Alvarez, 2010; Wang et al., 2003).

DNA repair is limited in spermatozoa and only occurs during specific stages of spermiogenesis. Repair mechanisms are necessary then to restore induced nicks and strand breaks that allow chromatin remodelling at this stage in haploid spermatozoa. Repair mechanisms are no longer activated during nuclear condensation in the epididymis (Leduc et al., 2008; Marcon and Boissonneault, 2004). Spermatozoa, however, are exposed to oxidative damage in the epididymis and during transport in seminal fluid. The next opportunity for DNA repair is by the oocyte, which is a critical step in embryo development. However, whereas DNA adducts induced by oxidative stress may be repairable by the oocyte, single- or double-stranded DNA breaks may not and they can have significant impact on fertilization and pregnancy outcome (Gonzalez-Marin et al., 2012; Menezo et al., 2007a).

Measuring DNA damage

Several methods are employed by routine diagnostic laboratories in the investigation of DNA fragmentation, most common among which are the sperm chromatin structure assay (SCSA), the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) and Comet assays and the sperm chromatin dispersion test (SCD) (Lewis et al., 2013) (**Table 1**).

According to the literature, the SCSA, giving a DNA fragmentation index (DFI) value, is perceived as the most statistically robust and reproducible test and is a valuable predictor of fertility (Bungum, 2012; Giwercman et al., 2010; Oleszczuk et al., 2011; Smit et al., 2007; Venkatesh et al., 2011). A valuable threshold for DFI was set by Evenson et al. (1999) at 30%, which is robustly indicative of fertility potential. This method also yields information about chromatin decondensation via measurement of high-density staining (i.e. staining of DNA over an intensity threshold indicates excessive access of the stain to the DNA via chromatin decondensation). The SCSA is based on acridine orange staining of DNA, whereby intact DNA fluoresces green and fragmented DNA fluoresces red (Chohan et al., 2006). The SCSA employs a flow cytometer, on which the samples are analysed immediately post incubation with acridine orange and up to 10,000 cells are counted. While the disadvantage here is the expense of a flow cytometer, there is little intratechnician variability (Shamsi et al., 2011). On the other hand, performing an acridine orange stain manually and counting using a fluorescent microscope

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Table 1 Methods for detection of DNA damage in spermatozoa.

Method	*Basis*	*Advantages*	*Disadvantages*
SCSA	Acridine orange staining and analysis using flow cytometry	Statistically robust, accepted thresholds, predictor of infertility, low variability, can detect chromatin decondensation	Flow cytometer is expensive, manual stain has high variability and may produce erroneous results
TUNEL	dUTP labelling of strand breaks	Reproducible when using flow cytometry, working thresholds, detects both single- and double-strand breaks	Flow cytometer is expensive, manual stain has high variability
COMET	Electrophoretic technique	Highly sensitive	Separate conditions required for detection of double-and single-strand breaks, difficult to optimize and standardize
SCD	Staining of DNA 'loop' in agarose	Simple and inexpensive, allows for analysis of sperm populations	Low numbers of spermatozoa analysed, some technician variability

SCD = sperm chromatin dispersion test; SCSA = sperm chromatin structure assay; TUNEL = TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling.

is subject to high variation and prolonged incubation time can cause erroneous results.

The TUNEL assay is a commonly used technique that incorporates biotinylated dUTP at 3′ ends of DNA strand breaks using template-independent TdT (Shamsi et al., 2008b). TUNEL can detect both single- and double-strand breaks and the labelled bases can be quantified using a bright field microscope, fluorescent microscope or flow cytometer. Again the flow cytometer is an expense but one that reduces intratechnician variability and issues with background staining. Intra-assay variability using the flow cytometer was shown to be <7% but the correlation with SCSA only gave a Pearson coefficient of 0.63 (Erenpreiss et al., 2004). Reliable thresholds for TUNEL require further confirmation, although a working threshold of 15–20% is considered high, correlating with reduced fertility potential (Benchaib et al., 2003, 2007; Greco et al., 2005c; Sharma et al., 2010).

The Comet assay is an electrophoretic technique based on the principle that smaller fragmented DNA will migrate faster towards the anode than intact DNA (Shamsi et al., 2008b). The Comet assay is an extremely sensitive technique that can detect both single- and double-stranded DNA breaks but under different conditions, alkali versus neutral respectively, which is a disadvantage. Double-strand breaks may be considered more vital to pregnancy outcome and thus the neutral Comet assay is most often used in relation to infertility. The alkali Comet assay can overestimate DNA damage due to alkali-labile sites, which decrease the sensitivity of this assay. However, there is vast interlaboratory variation and many modifications that need to be optimized to standardize this technique (Forchhammer et al., 2012).

The SCD test utilizes the ability of intact DNA to loop around the nucleus once embedded in agarose, giving a characteristic 'halo' appearance (Shamsi et al., 2011). The presence of single-strand breaks in the DNA prevents this halo from forming. Agarose gels are stained with eosin and azure B and the halos are analysed by eye. This is a simple and inexpensive technique but intra-individual variation in halo assessment and background staining are drawbacks. In one large study of men undergoing IVF/ICSI, the SCD was valuable in predicting fertilization rates with a threshold of 18%, but not of clinical pregnancy (Velez de la Calle et al., 2008). The SCD test was, however, not valuable in predicting outcome of intrauterine insemination (IUI; Muriel et al., 2006). While the numbers of spermatozoa analysed are much lower, this technique does allow for quantification of subpopulations such as highly DNA-degraded spermatozoa, the results of which may be valuable (Abad et al., 2013; Garcia-Peiro et al., 2012).

Impact on fertility

High levels of DNA fragmentation have been shown to be a robust indicator of male infertility (Evenson et al., 1999; Giwercman et al., 2010; Nicopoullos et al., 2008). As significant DNA fragmentation can be present in normozoospermic subfertile men, it may be an important factor in unexplained infertility (Erenpreiss et al., 2008; Oleszczuk et al., 2013). However, it also coexists with reduced count, increased abnormal forms (Chi et al., 2011; Mehdi et al., 2009; Moskovtsev et al., 2009; Virro et al., 2004) and notably with reduced motility (Appasamy et al., 2007; Erenpreiss et al., 2008; Giwercman et al., 2003; Lin et al., 2008). High DNA fragmentation has also been implicated in the aetiology of age-related infertility in the male (Das et al., 2013; Smit et al., 2010; Vagnini et al., 2007).

In the comprehensive study by Evenson et al. (1999), the threshold of DFI >30% indicated a marked and significant reduction in fertility potential (natural conception) including time to pregnancy. In a more recent analysis, Evenson and Wixon (2008) confirmed that the likelihood of a natural pregnancy with DFI >30% is very low. Success in IUI has also been shown in numerous studies to be very limited when DFI >30%. In a meta-analysis, Evenson and Wixon (2006) reported that couples were 7.3-times more likely to achieve pregnancy/delivery after IUI with DFI <30%. A large analysis of 998 cycles (387 IUI) from 637 couples demonstrated very limited success (1.5%) of IUI with DFI >30% (Bungum et al., 2007).

For IVF, high DFI (>30%) has been associated with a reduced number of quality embryos in some studies (Jiang et al., 2011; Niu et al., 2011; Zhang et al., 2008) although this may not have a significant effect on resulting pregnancy rates (Bungum et al., 2007; Niu et al., 2011). However, it

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has been repeatedly shown that ICSI outcome is independent of DFI and that clinical pregnancy rates are actually higher than with IVF (Bungum et al., 2004, 2007; Chi et al., 2011; Evenson and Wixon, 2006). Selecting for normal morphology and movement in ICSI can decrease the likelihood of fragmented DNA in fertilizing spermatozoa (Maettner et al., 2013; Sivanarayana et al., 2012). Using this selection process, a recent study has shown that success rates with ICSI with semen showing very high DFI (>50%) are similar to those with low DFI (<15%; Dar et al., 2013). It is therefore advised that couples contemplating assisted reproduction treatment with DFI >30% go directly to ICSI to avoid costly IUI and IVF failures.

However, ICSI with high DFI is not without its limitations. There is a significantly increased risk of miscarriage, implantation failure or failure to progress to delivery within this group (Benchaib et al., 2007; Bungum et al., 2007; Kennedy et al., 2011). Even with spontaneous conception, DNA damage is positively correlated with recurrent pregnancy loss (Brahem et al., 2011; Carrell et al., 2003; Gil-Villa et al., 2009; Kumar et al., 2012). Using ICSI bypasses many of the natural hurdles that spermatozoa undergo in order to fertilize an oocyte *in vivo*. The oocyte has limited capacity to repair such high levels of DNA fragmentation after fertilization during early embryo development and thus pregnancy is less likely to progress. Couples should be counselled as to the increased risk for biochemical pregnancy or early pregnancy loss under these circumstances. An additional concern is the future health of offspring. Paternal gametes are the major source of de-novo structural chromosome reorganizations (Thomas et al., 2010). Chromosomal abnormalities are higher in men selected for ICSI and can be passed down to offspring using this technique (Bonduelle et al., 2002; Mau et al., 1997; Meschede et al., 1998). Balanced structural reorganizations that allow successful pregnancy may result in phenotypically normal children but may cause infertility in these children if the reorganization is present in the gamete of the child (Egozcue et al., 2000). An often-cited example of the relationship between DNA damage and the health of the offspring can be found with paternal smoking. Paternal smoking can increase DNA fragmentation by 50% (Fraga et al., 1996) and is linked with increased incidence of childhood cancers (Ji et al., 1997; Shu et al., 1996). Thus men undergoing assisted reproduction treatment should aim to reduce DNA fragmentation regardless of the potential success rates offered by ICSI.

ROS generation in spermatozoa

Like somatic cells, spermatozoa produce small amounts of ROS as a byproduct of the electron transfer chain in mitochondria (Aitken and Clarkson, 1987; Koppers et al., 2008). The major ROS produced are O_2^- and H_2O_2, and antioxidants such as GPX, CAT and SOD, that neutralize these, are present both within mitochondria and in the secretions of the reproductive tract (Starkov, 2008; Vernet et al., 2004). Where there is excess ROS production, or in the absence of adequate antioxidants, concentrations of ROS may increase. H_2O_2 can also form the highly reactive and damaging $OH^{\cdot}$ radical in the presence of copper or iron (Haber–Weiss and Fenton reactions; Kehrer, 2000). Elevated concentrations of both copper and iron have been detected in seminal plasma of subfertile men (Aydemir et al., 2006). The $OH^{\cdot}$ radical is particularly damaging to DNA and lipids (Kehrer, 2000; Kwenang et al., 1987; Lloyd and Phillips, 1999). Mitochondrial DNA is also damaged by ROS which can limit ATP production and energy provision for motility in spermatozoa, reducing fertility (Shamsi et al., 2008a). Mitochondrial DNA mutations do not, however, impact the health of the offspring, as male mitochondrial DNA is degraded by the oocyte, leaving only maternal inheritance (Sutovsky et al., 2004).

Cytoplasmic generation of ROS in spermatic cells has also been suggested, although the exact mechanism is yet to be elucidated. During normal spermiogenesis, the majority of cytoplasm is extruded from the maturing spermatozoa by the action of Sertoli cells (Rengan et al., 2012). The remaining cytoplasmic droplet in the midpiece contains enzymes required for energy production such as creatinine kinase and glucose-6-phosphate dehydrogenase and it is here that the essential ROS production for capacitation occurs (Rengan et al., 2012). Glucose-6-phosphate dehydrogenase is required for the reduction of NADP + to NADPH, and it is proposed that this fuels ROS production by NADPH oxidase (Aitken et al., 1997; Dona et al., 2011a). It is thought that excess residual cytoplasm remaining from defective spermiogenesis increases the amount of glucose-6-phosphate dehydrogenase, leading to increased ROS production (Gomez et al., 1996). For this reason, immature spermatozoa, or 'incorrectly matured' spermatozoa, represent a major source of ROS in oxidative stress and may cause DNA damage in mature spermatozoa during cotransit in the epididymis (Gil-Guzman et al., 2001; Gomez et al., 1996; Ollero et al., 2001). In a study where spermatozoal subsets were isolated from the ejaculate, the number of immature spermatozoa directly correlated with levels of DNA damage in mature spermatozoa (Ollero et al., 2001).

Other sources of ROS and modifiable factors

Leukocytes

In addition to immature/abnormal spermatozoa, leukocytes are the major source of ROS and oxidative stress (Aitken and West, 1990; Whittington and Ford, 1999). Low numbers of leukocytes are present in the majority of normal ejaculates, and the WHO has defined leukocytospermia as $>1 \times 10^6$ spermatozoa/ml (WHO, 2010). However, the significance of this threshold is still debated. While studies have demonstrated a negative correlation of leukocyte concentration with sperm parameters (Aziz et al., 2004; Moskovtsev et al., 2007; Omu et al., 1999), others have shown that this may not be clinically relevant unless $>2 \times 10^6$ spermatozoa/ml (Yanushpolsky et al., 1996). In one study, leukocytospermia as defined by the WHO ($1-3 \times 10^6$ spermatozoa/ml) was shown to have a positive effect and to correlate with initiation of the acrosome reaction and hypo-osmotic swelling test (HOST) scores (Kaleli et al., 2000). Importantly though, in the context of this review, several studies have shown that leukocytospermia commonly coexists with elevated DNA damage in spermatozoa (Alvarez et al., 2002; Erenpreiss et al., 2002; Fariello et al., 2009; Saleh et al.,

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2002a). Increased seminal ROS associated with leukocytospermia coexists with reduced concentrations of antioxidants (Omu et al., 1999; Yadav et al., 2006). Thus, interindividual differences in the total seminal antioxidant capacity may explain variations in susceptibility to ROS damage from leukocytes and related dysfunction.

With infection or chronic inflammation, activated leukocytes greatly increase the concentrations of ROS and can release 1000-times more ROS than spermatozoa (Plante et al., 1994). These concentrations of ROS may overwhelm seminal fluid antioxidant defences. Not discounting this, the time spent in the ejaculate is relatively small and therefore infection of the epididymis or testes have the greatest significance (Ochsendorf, 1999). Thus it is clear that infection or inflammation should be treated accordingly in cases of male infertility. Notably, antibiotic treatment of low-level leukocytospermia ($0.2-1 \times 10^6$ spermatozoa/ml) resulted in a significant increase in spontaneous pregnancy and thus the threshold for treatment may need to be re-evaluated (Hamada et al., 2011).

Varicocele

Varicocele is the leading cause of male factor infertility, with an incidence of 15% in the whole male population and 40% among infertile men (Practice Committee of the American Society for Reproductive Medicine, 2004). Oxidative stress is a major factor in the pathophysiology (Agarwal et al., 2012) and elevated levels of DNA fragmentation have been demonstrated in numerous studies (Blumer et al., 2012; Saleh et al., 2003; Smit et al., 2013; Smith et al., 2006; Talebi et al., 2008; Zini and Dohle, 2011). Increased concentrations of ROS and decreased antioxidant capacity coexist in the majority of subjects (Abd-Elmoaty et al., 2010; Mostafa et al., 2012; Pasqualotto et al., 2008; Saleh et al., 2003; Smith et al., 2006). Interestingly some studies have shown elevated concentrations of antioxidants such as SOD and CAT (Altunoluk et al., 2012; Hurtado de Catalfo et al., 2007; Ozbek et al., 2008), which may account for an early increase in defences before becoming overwhelmed.

Although previously debated, it is now the consensus that varicocele, either symptomatic or as a cause of infertility, is best treated surgically and the gold standard is microsurgical repair (Ficarra et al., 2012; Goldstein and Tanrikut, 2006; Mehta and Goldstein, 2013). Surgical repair is associated with improved semen parameters, including DNA fragmentation, decreased ROS and increased antioxidants and with improved success with assisted reproduction, but above all with increased incidence of spontaneous conception in previously infertile men (Baker et al., 2013; Chen et al., 2008; Hurtado de Catalfo et al., 2007; Leung et al., 2013; Mostafa et al., 2001; Smit et al., 2013).

Smoking

Tobacco smoke contains high concentrations of ROS including O_2^- and $OH^{\cdot}$, shown to participate in Fenton reactions to produce H_2O_2 and cause DNA damage (Valavanidis et al., 2009). Cadmium and lead derived from cigarette smoke also cause DNA strand breaks (Hengstler et al., 2003) and are present in seminal fluid associated with indices of oxidative stress (Kiziler et al., 2007). Nicotine is oxidative and can induce double-stranded DNA breaks in sperm DNA *in vitro* (Arabi, 2004). Cotinine, its major metabolite, is detected in the seminal plasma of smokers (Wong et al., 2000).

In a small study (20 smokers excluding controls), smoking was associated with dramatic increases in seminal ROS concentrations (by 107%) and leukocyte numbers (by 48%) compared with nonsmokers (Saleh et al., 2002b). Smoking decreases overall concentrations of antioxidants in the body, indicating reduced protection against oxidative stress (Lesgards et al., 2002). Direct correlations with DNA damage indices have been demonstrated in a number of studies in fertile men (Linschooten et al., 2011; Taha et al., 2012), men with varicoceles (Fariello et al., 2012b) and men with idiopathic infertility (Elshal et al., 2009). The greatest difference was shown in the notable study of men with idiopathic infertility ($n = 70$): the mean DFI of infertile smokers was 37.66% compared with 19.34% in infertile nonsmokers ($P < 0.001$) or 14.51% in fertile controls (not significantly different from infertile nonsmokers). The correlation of DFI with smoking gave a Pearson coefficient of 0.796. This study carefully excluded confounding factors such as age, leukocytospermia, varicocele and other potential sources of DNA damage. This demonstrates the importance of smoking in the aetiology of idiopathic infertility.

As aforementioned, smoking has been linked with heritable DNA damage and incidence of childhood cancer. Cessation of smoking is therefore advised in the treatment of male factor infertility (**Table 2**).

Xenobiotics and toxic metals

Xenobiotics represent a diverse source of potential DNA damage. Infertile men under oxidative stress may have increased susceptibility, and a combined or additive effect may occur between different xenobiotics and other sources of ROS. It is for this reason that a thorough history and questionnaire is advised in treating male infertility so that exposure to such damaging chemicals can be reduced or avoided as part of a holistic approach.

Exposure to persistent organochlorine pollutants such as polychlorinated biphenyls and metabolites of dichlorodiphenyltrichloroethane may cause DNA fragmentation in spermatozoa. In studies of European men, exposure levels correlated with higher levels of DFI, while this was not observed in Inuit populations in Greenland where exposure would be higher (contaminated fatty fish) (Rignell-Hydbom et al., 2005; Spano et al., 2005; Stronati et al., 2006). However, genetic variation might be expected to confer protection in highly exposed populations. DFI has been shown to be markedly higher in exposed men with modifications of the androgen receptor gene (Giwercman et al., 2007). Sanchez-Pena et al. (2004) showed that 75% of Mexican workers exposed to organophosphorus pesticides had DFI >30% whereas unexposed controls had mean DFI 9.9%. Mean DFI of Venezuelan farmers exposed to organophosphorus and carbamate pesticides showed similar elevations to 34.8% ($P < 0.0001$) although unexposed controls had a higher DFI than the Mexican cohort (24.6%) (Miranda-Contreras et al., 2013). Occupational exposure to lead also correlates with DNA fragmentation in spermatozoa, even when adjusting for smoking (Hsu et al., 2009; Vani et al., 2012). Seasonally increased air pollution (sulphur dioxide, nitric oxide and

Table 2 Lifestyle factors modifiable without risk.

Lifestyle factor	*Results*	*Recommendations*	*References*
Smoking	Strong correlation with % DFI, DFI markedly higher in infertile smokers	Cessation of smoking	Elshal et al. (2009)
POP/PCB	Positive correlation between exposure and % DFI	PCB accumulate in food chain: avoid fatty fish, particularly farmed	Rignell-Hydbom et al. (2005), Spano et al. (2005), Stronati et al. (2006)
Organophosphorus	Marked increase in % DFI (>30%) in exposed workers	Avoid pesticide exposure	Sanchez-Pena et al. (2004)
Lead	Increase in percentage of spermatozoa with DNA fragmentation	Avoid occupational exposure and smoking or exposure to cigarette smoke	Hsu et al. (2009), Vani et al. (2012)
Bisphenol A	Significant trend of increased DNA damage with increased urinary bisphenol A concentrations	Avoid plastic packaging, tinned foods, heating or storing foods in plastic	Meeker et al. (2010)
Testicular heat	Increase in DNA fragmentation with 2–3°C temperature increase	Avoid cycling with tight pants, avoid sauna use, avoid using laptop on closed legs	Southorn (2002), Ahmad et al. (2012), Sheynkin et al. (2011), Garolla et al. (2013)
Mobile phone radiation	No specific studies on DNA fragmentation, increased ROS and decreased antioxidants	Do not store mobile phone in trouser pocket	Desai et al. (2009)
Obesity	Positive correlation of body mass index and DNA fragmentation, higher incidence in obese males	Weight loss through diet and moderate exercise	Kort et al. (2006), Chavarro et al. (2010), Fariello et al. (2012a), La Vignera et al. (2012), Dupont et al. (2013)

DFI = DNA fragmentation index; PCB = polychlorinated biphenyls; ROS = reactive oxygen species.

particulate matter) in the Czech Republic was associated with significantly increased DNA fragmentation during months of highest exposure, adjusting for smoking and other variables (Rubes et al., 2005).

Bisphenol A is an important endocrine disruptor and human exposure is widespread from food and drink containers and the environment (Rubin, 2011). Animal studies have demonstrated mutagenic effects, DNA damage induced in spermatozoa and epigenetic modifications in offspring (Dobrzynska and Radzikowska, 2013; Manikkam et al., 2013; Tiwari and Vanage, 2013). Limited studies have been carried out in humans. One study by Meeker et al. (2010) measured urinary bisphenol A and DNA damage by Comet assay in 132 men from subfertile couples. Adjusting for variables and other sources of DNA damage, a significant trend was seen for increasing Tail% DNA, which the authors attributed to single-strand breaks.

Some medications and drugs are associated with DNA damage in spermatozoa such as selective serotonin reuptake inhibitors (Safarinejad, 2008; Tanrikut et al., 2010) and opiates (Safarinejad et al., 2013). This reiterates the importance of taking an adequate history due to the high intake of these drugs, including over-the-counter drugs such as codeine. Avoidance of these xenobiotic sources would be advised in cases where exposure is evident (**Table 2**).

Heat

The position of the scrotum acts to maintain the temperature of the testes lower than that of the body. Elevation in scrotal temperature, or hyperthermia, is associated with male infertility and impaired spermatogenesis (Mieusset et al., 1987). Spermatogenic arrest increases the number of immature or 'incorrectly matured' spermatozoa in the epididymis and ejaculate (Dada et al., 2003), and these are a major source of ROS (Gil-Guzman et al., 2001; Ollero et al., 2001). Scrotal hyperthermia may result from close-fitting underwear although the degree of hyperthermia caused by briefs rather than boxer shorts is debated as somewhat of a myth (Southorn, 2002). Specially designed tight-fitting underwear that maintained the testes at inguinal position did elevate scrotal temperature by 2°C in one study (Ahmad et al., 2012). This study demonstrated a significant increase in both DNA fragmentation and chromatin decondensation if sustained for 20 days or more at this temperature increase. Cycling has also been proposed as a

source of hyperthermia. Moderate cycling under tightly controlled laboratory conditions was shown not to cause scrotal hyperthermia (Jung et al., 2008). However, the subjects of this study were wearing loosely fitted cotton trousers whereas most cyclists wear tight-fitting lycra, which is proposed to be the source of hyperthermia (Southorn, 2002). Regular use of a sauna, Jacuzzi or hot bath is also contraindicated. In a small study of 10 normozoospermic healthy volunteers, there was a temperature increase from 34.5°C to 37.5°C with regular sauna use (Garolla et al., 2013). This usage resulted in nonsignificant increases in DNA fragmentation (by both TUNEL and acridine orange assay) and a significant increase in chromatin decondensation. Incidence of acute febrile illness is also associated with impaired semen parameters and excludes men from routine tests, trials and donation for assisted reproduction. Fever has been shown to significantly increase DNA fragmentation for a period lasting at least 79 days, peaking at approximately 1-month post illness (Sergerie et al., 2007). Finally, positioning a laptop on the lap of closed legs markedly increases scrotal temperature (Sheynkin et al., 2011). This and other sources of scrotal hyperthermia should be avoided in men that are trying to conceive, particularly men diagnosed with elevated DNA fragmentation (**Table 2**).

Mobile phone radiation

Increased usage of mobile phones and storage of phones in trouser pockets has been suggested as a source of damaging radiation to the male reproductive system (**Table 2**). A recent study showed that DNA fragmentation was the only parameter altered in mobile phone users, in a group of high usage (>4 h daily) that stored their phone in the trouser pocket (Rago et al., 2013). Previous studies have shown alterations in motility, morphology and hormonal disturbances (Agarwal et al., 2008; Fejes et al., 2005; Gutschi et al., 2011). Mobile phone radiation can increase ROS production and decrease activity of CAT, SOD and GPX (Desai et al., 2009). DNA damage may result from this, as has been clearly demonstrated *in vitro* (De Iuliis et al., 2009a).

Age

Age-related infertility has been linked with DNA damage where age correlates positively with DNA fragmentation (Das et al., 2013; Hammiche et al., 2011; Rybar et al., 2011). Because age is a nonmodifiable variable in the treatment of male infertility, it is outside the scope of this review to discuss related mechanisms.

Obesity

Obesity is associated with male factor infertility and abnormal semen parameters mainly due to hormonal aberrations and incidence of negative lifestyle factors (Du Plessis et al., 2010; Hammoud et al., 2008). DNA damage has also been investigated in numerous studies of obese men. One study of 305 subfertile men ($n = 36$ obese) demonstrated a significant increase in DNA fragmentation as measured by the Comet assay with obesity (Fariello et al., 2012a). Analysis of men of subfertile couples (possibly not all men were subfertile) also showed a higher incidence of spermatozoa with DNA fragmentation in obese men (Chavarro et al., 2010; Dupont et al., 2013). A study of 520 men presenting for semen analysis demonstrated a positive correlation between body mass index and DFI, with the mean DFI rising from 19.9% in normal body mass index men to 27.0% in obese men (Kort et al., 2006). Finally, a study of normal volunteers from the general population ($n = 50$ obese) also confirmed the relationship between body mass index and DNA damage as measured by TUNEL (La Vignera et al., 2012). Moderate exercise combined with diet may be recommended for weight loss (**Table 2**). Both moderate exercise and weight loss have correlated with improved semen parameters in a limited number of studies (Reis and Dias, 2012; Sharma et al., 2013), although there have been no human studies to date that measured DNA fragmentation as an outcome.

Antioxidants

Antioxidants from the diet can act directly as antioxidants or are essential cofactors to the enzymic antioxidant systems SOD, CAT and GPX. As reviewed by Lanzafame et al. (2009), seminal plasma contains both enzymic antioxidants and low-molecular-weight nonenzymic antioxidants such as ascorbic acid, α-tocopherol, uric acid, albumin, carnitine, carotenoids, flavonoids and coenzyme Q10 (CoQ10) (Lanzafame et al., 2009).

Dietary antioxidant intake

Food frequency questionnaires have been utilized to elucidate the association between dietary intakes and sperm parameters, including DNA damage. In one study, older men (>44 years) with greater intakes of vitamin C, vitamin E and zinc had the lowest levels of DNA fragmentation, similar to levels in younger men (Schmid et al., 2012). In a study of men attending an assisted reproduction clinic in the Netherlands ($n = 161$), a 'health conscious' diet was

Table 3 Dietary sources of antioxidants.

Vitamin C	*Vitamin E*	*Zinc*	*Selenium*
Papaya	Spinach	Spinach	Halibut
Bell peppers	Swiss chard	Shiitake mushroom	Tuna
Strawberries	Sunflower seeds	Crimini mushroom	Cod
Broccoli	Almonds	Organic lamb	Shrimp
Pineapple	Asparagus	Organic beef	Crimin mushroom
Kiwi	Bell peppers	Scallops	Mustard seeds
Oranges	Cayenne pepper	Sesame seeds	Sardines
Canataloupe	Papaya	Pumpkin seeds	Salmon
Kale	Kale	Oats	Turkey
Cauliflower			Barley

Adapted from World's Healthiest Foods (2013).

Table 4 Comparison of studies of antioxidant treatment in DNA fragmentation.

Study	*Supplement*	*Measurement*	*Study design*	*Outcome*
Fraga et al. (1991)	Vitamin C	8-OHdG	10 normal volunteers, uncontrolled, depletion to 5 mg/day, repletion to 250 mg/day	DNA damage increased upon depletion, restored by repletion
Greco et al. (2005a)	1 g vitamin C, 1 g vitamin E	TUNEL	64 males with >15% damage, placebo controlled, double blinded	DNA damage reduced from 22.1% to 9.1%
Greco et al. (2005b)	1 g vitamin C, 1 g vitamin E	TUNEL	38 infertile males,>15% damage, uncontrolled	DNA damage reduced from 24.8% to 8.2% in responsive group
Kodama et al. (1997)	400 mg GSH, 200 mg vitamin C, 200 mg vitamin E	8-OHdG	14 males with DNA damage, uncontrolled	Modest decrease in DNA damage; possible marked decrease in responsive group
Menezo et al. (2007b)	400 mg vitamin C, 400 mg vitamin E, 33 mg zinc, 80 μg selenium, 18 mg β-carotene	SCSA	58 males with DFI >15%, double centred, uncontrolled	DFI decreased from 32.4% to 26.2%, high-density staining increased
Tremellen et al. (2007)	Menevit: 100 mg vitamin C, 400 IU vitamin E, 6 mg lycopene, 333 μg garlic oil, 25 mg zinc, 26 μg selenium, 500 μg folic acid	TUNEL	60 males with damage >25%, double-blind RCT	DNA damage reduced from 37.9% to 33.3%; but from 40.03% to 32% in controls[a]
Tunc et al. (2009)	Menevit: as above	TUNEL	50 males with seminal oxidative stress, uncontrolled	DNA damage reduced from 22.2% to 18.2%
Abad et al. (2013)	1500 mg L-carnitine, 20 mg CoQ10, 60 mg vitamin C, 10 mg vitamin E, 200 μg vitamin B9, 1 μg vitamin B12, 10 mg zinc, 50 μg selenium	SCD	20 asthenteratozoospermic males, uncontrolled	DNA damage reduced from 28.5% to 20.12%

8-OHdG = 8-hydroxy-2-deoxyguanosine; DFI = DNA fragmentation index; GSH = glutathione; RCT = randomized controlled trial; SCD = sperm chromatin dispersion test; SCSA = sperm chromatin structure assay; TUNEL = TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling.
[a]Results from patent submission for Menevit supplement (Tremellen, 2006).

significantly associated with a lower risk of DNA fragmentation, specifically attributed to higher intakes of fruits and vegetables, which are excellent sources of antioxidants (Vujkovic et al., 2009); the study was adjusted for body mass index, smoking, age and vitamin supplementation. In a study of normal volunteers, the same association was not seen: there was no correlation between intakes of vitamin C, E and β-carotene and % DFI (Silver et al., 2005). The authors note that this does not preclude the potential effects of antioxidant status on subfertile men.

Diet is a major modifiable factor in the treatment of male infertility. Antioxidants undoubtedly play an important role in spermatozoa health, and dietary changes should be considered in any treatment regimen involving elevated DNA fragmentation (**Table 3**).

Supplementary antioxidants

Antioxidant therapy for DNA fragmentation has been tested in numerous trials. The overall view is that antioxidant treatment may play a relevant role, although some results have been inconsistent and large well-controlled trials are still required for conclusive evidence as to their efficacy (Gharagozloo and Aitken, 2011; Lombardo et al., 2011; Showell et al., 2011; Zini et al., 2009). There is large heterogeneity to studies including design, antioxidants used singly or in combinations, cohort selection (aetiology of infertility) and outcome measurement. Many reviews have called out for relevant clinical outcomes to be included such as pregnancy or delivery rate. However, in the field of

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Table 5 Modifiable factors in the treatment of oxidative stress causing DNA fragmentation.

Medical factor	*Lifestyle change*	*Dietary change*	*Supplement*
Treatment of leukocytospermia	Smoking cessation	Healthy diet	Vitamin C
Surgical repair of varicocele	Avoid xenobiotic sources of reactive oxygen species	Increase in fruit/vegetables and sources of antioxidants	Vitamin E
	Avoid heavy/toxic metals	Weight loss	Mixed antioxidants?
	Avoid testicular heat		
	Avoid testicular mobile phone radiation		

fertility, there are many variables that affect delivery rates in natural pregnancy and, particularly, after assisted reproduction treatment. For this review, we chose to only assess studies that directly measured the effect of antioxidants on DNA fragmentation in order to limit variable factors and to determine the effect, if any, of antioxidant intake on DNA damage in spermatozoa (**Table 4**).

Vitamin C

Vitamin C is a powerful hydrophilic antioxidant in seminal plasma. The incidence of DNA fragmentation is higher in men with low concentrations of seminal ascorbic acid (Song et al., 2006) while the incidence is lower in men with higher dietary intake (Schmid et al., 2012). One notable study examined the effects of ascorbic acid concentrations on DNA damage using measurement of 8-OHdG concentration. Concentrations of ascorbic acid in 10 normal individuals were maintained via a controlled diet (Fraga et al., 1991). While this was a very small study with no control group, it is of interest due to the model of depletion and repletion utilized. During an initial 32-day period of depletion, 8-OHdG concentrations almost doubled. This increase was followed by a 28-day period of marginal repletion where 8-OHdG further increased to 2.5-fold higher than the baseline value. Full repletion over 28 days then resulted in a reduction of DNA damage by 49%, although a period of 1 month may not have been sufficient to see the full effect. Of additional note in this study was that oral intake rapidly affected concentrations of ascorbic acid in seminal plasma. Upon depletion, seminal concentrations reduced by 71%, which was fully restored after the period of repletion.

In another study, DNA fragmentation (Comet assay) was prevented in lead-exposed workers in India by administration of 1000 mg vitamin C 5 days/week for 3 months when administered prophylactically (Vani et al., 2012). However, high doses of vitamin C may be linked with negative effects. Ascorbate can reduce disulphide bridges of cysteine residues (Giustarini et al., 2008), potentially damaging the chromatin structure. A study of mixed antioxidants, including just 400 mg of ascorbic acid daily for 3 months, attributed this effect to the induction of chromatin decondensation in spermatozoa (Menezo et al., 2007b). In order to avoid oversupplementation, blood tests can be used to determine plasma concentrations of vitamin C, although seminal plasma concentrations are far higher (~600 μmol/l compared with ~40 μmol/l (Frei et al., 1989; Thiele et al., 1995)). Bioavailability from food sources including fruit and vegetables is good, and intake can restore suboptimal plasma concentrations rapidly (Vissers et al., 2013).

Vitamin C and E

Oral vitamin E supplementation affords an increase in both plasma and seminal concentrations, with a nonsignificant difference found between intakes of 600, 800 and 1200 mg, favouring 800 mg (Moilanen and Hovatta, 1995). With regard to effects on DNA fragmentation, the only studies investigating vitamin E have used mixed antioxidants. Vitamin C and E can work synergistically, although ascorbate is present in much greater amounts in seminal fluid (Lewis et al., 1997). The first publication using mixed antioxidants was a convincing placebo-controlled double-blind study (Greco et al., 2005a). Men with varicocele, infection or inflammation and smokers were excluded. A 2-month period was used to assess post-testicular DNA damage. The mean DNA damage was markedly reduced from 22.1% to 9.1% in the treatment group, with no change in the placebo group. The second publication had a similar treatment design but no placebo group (Greco et al., 2005b). Men were treated after one failed ICSI attempt and a second attempt was carried out immediately after antioxidant treatment. This study divided the group into 'responsive' and 'nonresponsive'. Those responsive to antioxidant treatment (29/38, 76%) had a decrease in DNA damage from a mean of 24% to 8.2%, similar to the first study. It was suggested by the authors that alternative pathologies may be present in the 'nonresponsive' group and that this may also represent a source of variation in other studies, contributing to the heterogeneity of results seen in this field. It could also be argued that antioxidant treatment should be based on the individual rather than a blanket treatment regimen. With this approach, these trials suggest a very positive role for vitamin C and E in the treatment of DNA fragmentation. In addition, the clinical pregnancy rate increased remarkably and significantly after treatment, from 2/29 (both which ended in spontaneous abortion) to 14/29 (48%).

A previous small study combined vitamin C and E with glutathione (Kodama et al., 1997), although oral glutathione has poor bioavailability (Allen and Bradley, 2011). This study demonstrated a modest decrease in 8-OHdG values in infertile men after 2 months. The authors suggest that lack of controls and high interpatient variability do not conclusively show efficacy. However, the data shows a 50% response rate, whereby there was a more notable decrease in oxidative damage in seven participants, including the male with

the highest 8-OHdG value. If the group was divided into 'responsive' and 'nonresponsive' in a manner similar to Greco et al. (2005b), the results may have shown a greater mean decrease in responders alone. In addition, the quantities of vitamins were much lower in this study and so the modest response may still be relevant.

Zinc and selenium

Zinc is an integral element in the development of spermatozoa and DNA synthesis and an important antioxidant in seminal fluid (Ebisch et al., 2007). It can prevent iron- and copper-mediated lipid peroxdiation and works synergistically with α-tocopherol in this regard (Zago and Oteiza, 2001). It is also required for Cu/Zn superoxide dismutase, which is the type of SOD found in high amounts in the male reproductive tract (Mruk et al., 2002). Substantial zinc deficiency is common worldwide (Wessells and Brown, 2012). Testing for blood concentrations of zinc may identify deficiency as seen in oligozoospermic and azoospermic men (Ali et al., 2005), although in some cases concentrations may be normal in blood but decreased in seminal fluid under local conditions of oxidative stress (Chia et al., 2000). Testing for seminal oxidative stress is not routine and protocols need to be established and tested before this becomes a reliable parameter on which to base antioxidant therapy (Deepinder et al., 2008). Hair mineral analysis may be useful for both zinc and selenium status assessment, although results require careful interpretation and more robust trials are required to prove its reliability (Ashton et al., 2009; Lowe et al., 2009; Namkoong et al., 2013).

Selenium is an essential component of the GPX selenoproteins (Tinggi, 2008). Outright selenium deficiency is rare in the USA (USDA, 2009/2010). Conversely, the National Diet and Nutrition Survey in the UK showed that intakes were approximately 70% of the recommended nutrient intake, with half of women and a fifth of men below the lower recommended limit (Food Standards Agency and Department of Health, 2008/2009). Intake from food can also be underestimated. Widespread deficiency of selenium in soil can result in lower selenium concentrations in foodstuffs such as meat, with daily intakes varying greatly across countries (European Commission Scientific Committee on Food, 2000; Niskar et al., 2003).

A study of mixed antioxidants including zinc and selenium by Menezo et al. (2007b) demonstrated a significant reduction of DNA fragmentation, but a worrying increase in high-density staining, indicating chromatin decondensation. After 3 months of supplementation the mean DFI decreased from 32.4% to 26.2%, below the important threshold of 30%. However, high-density staining increased from 17.5% to 21.5%, which was attributed to the ability of vitamin C to reduce disulphide bridges, as already discussed. The authors themselves have observed a threshold of 28% for high-density staining beyond which no pregnancy can be achieved. Thus they recommend that antioxidant treatment is not given to men with high-density staining >20% while below this, the risk–benefit ratio is in favour of antioxidants. It could also be argued that, while zinc and selenium may be beneficial, limiting the amount of vitamin C could prevent the increase in chromatin decondensation.

Two additional studies have been published by an Australian group using the Menevit mixed supplement formulation, which includes vitamins C and E, zinc and selenium (Tremellen et al., 2007; Tunc et al., 2009). The first study by Tremellen et al. (2007) was a double-blind randomized control trial. The outcomes reported in this publication included fertilization, embryo quality and pregnancy success. There was a significant increase in pregnancy success in the treatment group, with clinical pregnancy detected in 38.5% of embryos transferred versus 16% in the control group. The measurements of DNA fragmentation were not reported in this publication but a patent submitted for the Menevit supplement gave further information (Tremellen, 2006). There was a nonsignificant reduction of DNA fragmentation in the treated group from 37.9% to 33.3% but a greater reduction was seen in the placebo group, from 40.3% to 32%. This was attributed to the statistical 'regression-towards-the-mean' phenomenon. In any case, the decrease in DNA fragmentation cannot explain the increase in pregnancy rates in this study.

The subsequent study by this group claim to avoid the 'regression-towards-the-mean' phenomenon by selecting men with proven levels of seminal oxidative stress rather than those chosen for extremes of the primary outcome measured (Tunc et al., 2009). This trial was uncontrolled and, given the results of the previous study by the same group, the evidence presented was weak. In any case, the mean decrease in DNA fragmentation was from 22.2% to 18.2%. Interestingly, however, a subgroup of participants whose partners subsequently became pregnant after IVF/ICSI (13/36, 36%) did have a more marked response, from 22% to 13.3%, indicating a possible link between improvement of DNA fragmentation and pregnancy outcome.

L-Carnitine and coenzyme Q10

Carnitines are found at high concentrations in the epididymis and are key to spermatozoal maturation and metabolism (Agarwal and Said, 2004). L-Carnitine is also a powerful antioxidant, which can prevent lipid peroxidation, scavenge O_2^- and H_2O_2 and inhibit iron-mediated ROS production (Gulcin, 2006). A systematic review by Zhou et al. (2007) indicated that supplementation can improve pregnancy rates and more specifically motility. CoQ10 is a strong antioxidant in its reduced form (ubiquinol) and can prevent oxidative damage to lipids and DNA (Littarru and Tiano, 2007). Specifically in seminal fluid, a significant negative correlation between ubiquinol concentrations and lipid peroxidation products has been demonstrated (Alleva et al., 1997).

Only one study was found which included L-carnitine and CoQ10 in the investigation of DNA fragmentation (Abad et al., 2013). DNA fragmentation was significantly reduced from 28.5% to 20.12%. While this study was uncontrolled, concomitant improvements in other semen parameters suggest that this therapy was successful in this cohort rather than being an artefact. However, as with mixed antioxidants, there is no way to determine which played a significant role in altering the parameters.

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Recommendations

As summarized in Table 5, medically modifiable factors such as leukocytospermia and varicocele should be assessed and addressed by the appropriate physician. Lifestyle factors are easily modifiable without risk in men presenting with DNA fragmentation. Lifestyle changes can therefore be readily recommended even in the absence of conclusive evidence. Dietary changes may also be carried out under the supervision of a dietician or nutritional therapist, assessing the current intakes of antioxidants particularly and incorporating foods high in vitamin C, vitamin E, zinc and selenium. Supplements should also be advised where deficiency has been detected by relevant testing. Antioxidant therapies should always be individually tailored according to deficiency or risk. DNA fragmentation should be retested after a 3-month period of treatment to allow for a full cycle of spermatogenesis.

Conclusion

DNA fragmentation is underevaluated in male infertility, but represents an extremely important parameter indicative of infertility and potential outcome of assisted reproduction treatment. It is also important to consider the health of offspring produced by assisted reproduction procedures that bypass natural hurdles such as ICSI, allowing men with high levels of DNA damage to reproduce. Oxidative stress is the major cause of DNA fragmentation in male infertility but may be modifiable in many cases. Sources of oxidative stress as discussed herein should be analysed in men exhibiting DNA fragmentation and avoided or limited as part of a treatment protocol. Using antioxidant supplementation has shown an overall benefit, most especially vitamins C and E, but caution is advised whether because of the risk of oversupplementing or of supplementing any nutrients without determining deficiency or necessity due to the delicate balance of reduction and oxidation required for fertility and ultimately successful fertilization and ensuing pregnancy.

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