The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy

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Abstract: Oxidative stress in the male germ line is thought to affect male fertility and impact upon normal embryonic development. Accordingly, fertility specialists are actively exploring the diagnosis of such stress in spermatozoa and evaluating the possible use of antioxidants to ameliorate this condition. In this review, evidence for the presence of oxidative stress in human spermatozoa, the origins of this phenomenon, its clinical significance in the aetiology of male infertility and recent advances in methods for its diagnosis and treatment are re-examined. Moreover, an extensive review of the results presented in published clinical studies has been conducted to evaluate the overall impact of oral antioxidants on measures of sperm oxidative stress and DNA damage. Administration of antioxidants to infertile men has been assessed in numerous clinical studies with at least 20 reports highlighting its effect on measures of oxidative stress in human spermatozoa. A qualitative but detailed review of the results revealed that 19 of the 20 studies conclusively showed a significant reduction relating to some measure of oxidative stress in these cells. Strong evidence also supports improved motility, particularly in asthenospermic patients. However, of these studies, only 10 reported pregnancy-related outcomes, with 6 reporting positive associations. Adequately powered, placebo-controlled comprehensive clinical trials are now required to establish a clear role for antioxidants in the prevention of oxidative stress in the male germ line, such that the clinical utility of this form of therapy becomes established once and for all.

Key words: human spermatozoa / reactive oxygen species / oxidative stress / antioxidant therapy

Introduction

Cellular oxidative stress becomes manifest when oxidants overwhelm the antioxidant defence system in cells. The excess oxidants can take part in specific and non-specific reactions with nearby cellular components such as unsaturated lipids, proteins and DNA, consequently impairing normal cellular processes. Cellular oxidative stress can arise from a high turnover of oxidants by cells or be due to the low levels of enzymatic and non-enzymatic antioxidant defence molecules. For reasons not well understood at the molecular level, most cell types can develop an early onset of oxidative stress phenomenon or a ‘state of predisease’. Accordingly, oxidative stress has been implicated in many major disease states, including cardiovascular disease, cancer, diabetes and brain disorders (e.g. Alzheimer’s), as well as male and female infertility. According to MEDLINE, in the last decade alone ‘oxidative stress’ has been mentioned in about 80 000 publications with over 700 papers reporting the phenomenon specifically in spermatozoa. Interestingly, spermatozoa were the first cell type reported to show a potential susceptibility to oxidative damage. In a landmark paper published in 1943, MacLeod (1943) confirmed a rapid loss of motility if spermatozoa were incubated in an oxygen-rich environment. Believing the loss of motility was due to an overproduction of oxidants arising from increased oxygen metabolism by sperm, he added the antioxidant, catalase, to the medium and restored motility, thereby successfully validating his hypothesis.

Numerous subsequent studies have confirmed the generation of a whole array of oxidants by different cell types. They are commonly referred to as reactive oxygen species (ROS) and include hydroxyl radicals (OH), superoxide anion (O2−) and hydrogen peroxide (H2O2). Several studies have reported a significant rise in ROS activity by human spermatozoa in various types of male infertility (Aitken and Clarkson, 1987; Aitken et al., 1989b, 1991, 1992; D’Agata et al., 1990; Iwasaki and Gagnon, 1992; de Lamirande et al., 1995; Sharma and Agarwal, 1996 Kodama et al., 1997; Shen et al., 1999; Zini et al., 1993) and it is now widely, if not universally, agreed that excess ROS contributes significantly to sperm DNA damage and lipid peroxidation (Alvarez et al., 1987; Hughes et al., 1996; Aitken and De Iuliis, 2010; Aitken et al., 2010). DNA damage in spermatozoa is, in turn, linked to poor rates of...
fertilization, impaired embryonic development, pregnancy loss, birth defects (Sukcharoen et al., 1993; Hansen et al., 2002; Carrell et al., 2003; Baker and Aitken, 2005; Lewis and Aitken, 2005) and the manifestation of diverse forms of morbidity in the offspring, including autism and childhood cancer (Ji et al., 1997; Crow, 2000; Sipos et al., 2004; Vestergaard et al., 2005; Reichenberg et al., 2006; Aitken et al., 2009). This is of great concern since a recent study of semen samples obtained from some 1600 men attending fertility institutes in France revealed moderate to high levels of sperm DNA damage in about 60% of the patients (Cohen-Bacrie et al., 2009). Despite the potential risks, the impact of DNA damage and its clinical significance remains somewhat controversial and most IVF physicians remain sceptical over diagnosis and treatment (Practice Committee of American Society for Reproductive Medicine, 2008).

This controversy is mainly due to a paucity of clinical studies that consistently demonstrate, across all data sets, the significance of oxidative stress in the diagnosis of male infertility (Makhlouf and Niederberger, 2006) and the importance of antioxidants in the management of this condition (Zini et al., 2009; Ross et al., 2010). In terms of the effectiveness of antioxidant therapy, inconsistencies in the literature reflect the inherent complexity of the reproductive process, variations in patient selection and sample sizes, inadequate study designs, the effectiveness (or otherwise) of DNA repair mechanisms in the oocyte, the use of non-standardized assays to detect DNA damage, as well as large differences in antioxidant doses and durations of treatment. As the lack of consensus over the importance of oxidative stress in male infertility and its treatment persists, increasing numbers of infertile men, particularly those with repeated IVF failures, consider self-medicating with antioxidants or antioxidant formulations. As a result, over the last decade, a small industry has burgeoned around the use of antioxidant nutraceutical formulations with at least 15 such formulations now available in USA alone. Interestingly, they differ substantially in the variety of antioxidant ingredients and doses used. No credible human clinical data are reported for any of them. Worryingly, some of these formulations combine a large number of antioxidants with aggressive doses, raising the possibility of ‘reductive stress’ by potentially depleting the physiological levels of ROS known to be critical for normal sperm function (O’Flaherty et al., 2005). Current regulations in the USA require no food and drug administration approval and allow the commercial availability of such formulations as long as direct explicit disease claims are not made.

Thus, under the current status quo, the majority of infertile men are not diagnosed or adequately treated for sperm oxidative stress. The availability of so many antioxidant formulations is a testament to the fact that a significant number of infertile men are now taking such formulations arbitrarily. Standardized, clinically well-proven, diagnoses followed by adequately substantiated pharmacotherapy of sperm oxidative stress and DNA damage must therefore be established as a matter of urgency. In this paper, the authors re-examine the underlying causes of sperm oxidative stress and DNA damage and consider its clinical significance in the aetiology of male infertility. Although the importance of oxidative stress in the aetiology of sperm DNA damage has been the subject of previous reviews, this article focuses particularly on the rationale behind the use of antioxidants to treat this condition and examines the clinical data regarding the effectiveness of such a therapeutic strategy.

An overview of sperm oxidative stress and DNA damage in male infertility

The presence of the high levels of ROS in various forms of male infertility has been well documented (Shen et al., 1999; Agarwal et al., 2008; Aitken et al., 2010). The involvement of ROS in male infertility comes from its capacity to induce detrimental chemical and structural modifications to sperm nuclear DNA as well as damage to the proteins and lipids in sperm plasma- and mitochondrial-membranes. The sperm plasma membrane is particularly vulnerable to oxidative stress because it is richly endowed with polyunsaturated fatty acids such as docosahexaenoic acid, which—with six double bonds per molecule—creates an ‘electron sink’ that renders it highly susceptible to oxidation and other chemical and structural modifications (Jones et al., 1978, 1979). ROS modification of the sperm plasma membrane disrupts its fluidity, leading to motility loss and impaired membrane fusion events such as the acrosome reaction and sperm–oocyte fusion (Aitken et al., 1989a). ROS are also known to cause significant DNA damage to both the mitochondrial and nuclear genomes of human spermatozoa (Sawyer et al., 2003). In such instances, these reactive oxygen metabolites attack DNA bases (particularly guanine), and phosphodiester backbones, destabilizing this molecule and creating the cellular conditions that ultimately result in DNA fragmentation (Kemal Duru et al., 2000; Wang et al., 2003; Villegas et al., 2005). Numerous studies have now established a strong association between oxidative stress and sperm DNA damage using techniques such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), sperm chromatin structure assay (SCSA) and measurement of the DNA oxidation adduct, 8-hydroxydeoxyguanosine (8-OHdG) (Aitken et al., 1998; Oger et al., 2003; Henkel et al., 2005; Kao et al., 2008; De Iuliis et al., 2009b). The degree and spectrum of oxidative damage to spermatozoa will depend upon the local environment and the nature of stress-inducing agents, which can be endogenous or exogenous and emanate from a wide range of pathological and environmental stressors (Fig. 1).

The principal sources of endogenous ROS in semen are leukocytes (Aitken et al., 1994, 1995; Aitken and Baker, 1995) and abnormal spermatozoa (Gomez et al., 1996; Sakkas et al., 2003). Every human semen specimen is contaminated by leukocytes, mainly neutrophils and macrophages. Stimulated neutrophils are professional generators of toxic reactive oxygen intermediates, particularly O2•− and H2O2. Co-released myeloperoxidases in these cells use H2O2 to oxidize chloride to hypochlorous acid (HOCl), a renowned highly potent oxidant that is produced by neutrophils in appreciable quantities (Vile et al., 2000). HOCl can react with endogenous amines to yield chloramines (Winterbourn, 1985) that are mutagenic (Weitzman and Stossel, 1981; Thomas et al., 1987) and cytotoxic (Thomas et al., 1983; Dallegrä et al., 1986). Neutrophil-derived HOCl was recently reported to induce changes reminiscent of apoptosis in human spermatozoa (Lessig et al., 2005). Exposure of calf thymus DNA to HOCl induces extensive DNA base modification, including formation of chlorinated bases (Whiteman et al., 1997). Oxidative stress invoked by leukocytes should be of particular concern in infection that is chronic (Kullisaar et al., 2008) or epidemic (Haidl et al.,...
in origin, as it has been associated with the induction of significant sperm DNA damage (Alvarez et al., 2002).

Functionally defective spermatozoa are another major source of ROS production (Gomez et al., 1996; Gil-Guzman et al., 2001; Aitken et al., 2003b). A plausible hypothesis recently suggested is that in most cases, the sperm DNA is attacked mainly by mitochondrial ROS originating from functionally defective spermatozoa (Koppers et al., 2008, 2010; Aitken and De Iuliis, 2010). Excessive mitochondrial production of ROS from these cells is known to correlate well with defective sperm function, particularly human sperm motility (Koppers et al., 2008). The mechanisms responsible for the activation of mitochondrial ROS generation are unknown, but any factor capable of interfering with the redox properties of these organelles is a potential inducer of ROS and DNA damage. Examples of such compounds are not only pharmacological mitochondrial inhibitors such as antymycin A and rotenone but also the presence of excessive quantities of polyunsaturated fatty acid (Koppers et al., 2008, 2010). Several studies also implicate poor chromatin remodelling in the origin of sperm DNA damage (Zini et al., 2007; De Iuliis et al., 2009b). Spermatozoa exhibiting low levels of nuclear protamination are highly susceptible to DNA oxidative attack due to greater accessibility of their DNA bases and backbone to ROS. The reasons for impaired protamination are unknown but some studies link this phenomenon to steroid-induced suppression of follicle stimulating hormone or luteinizing hormone (Aleem et al., 2008) or age (Zubkova and Robaire, 2006; Piazza et al., 2007). Exposure to toxic alkylating agents such as cyclophosphamide (Codrington et al., 2007), additional chemotherapeutic agents (Spermon et al., 2006) as well as other environmental toxicants that are capable of alkylating the free thiol on sperm protamines may also significantly contribute to the impairment of chromatin compaction, thus enhancing the susceptibility of these cells to oxidative attack (Sega, 1991).

The contribution of exogenous factors to protein and DNA damage in spermatozoa may therefore be significant.

The US Environmental Protection Agency now lists some 80 000 chemicals in human use, but only a small percentage has been tested for long-term safety. Among these, xenobiotics that redox cycle and/or are genotoxic in nature have become of particular concern to male reproductive health (Robaire and Hales, 2003; Aitken et al., 2004). A wide variety of xenobiotics with aromatic rings or conjugated bonds can be reduced enzymatically to form free radicals. Once formed, they can reduce molecular \( \mathrm{O}_2 \) to \( \mathrm{O}_2^- \) to regenerate the parent compound. The increased formation of superoxide anion can then create a state of oxidative stress through a complex series of secondary reactions that result in damage to the sperm plasma- and mitochondrial-membranes and nuclear DNA. Examples of redox cycling-inducing molecules are the viologens such as the world’s most widely used herbicide, Paraquat (Bus and Gibson, 1984; Hossain et al., 2010) and quinones such as menadione (Bennetts et al., 2008). Directly acting genotoxins, on the other hand, are xenobiotics (or their metabolites) that can form strong covalent bonds with DNA through electrophilic or nucleophilic addition reactions, resulting in the formation of various xenobiotic-DNA adducts, thus preventing accurate replication. Examples of xenobiotics acting as electrophiles or nucleophiles are the pesticide 1,2-dibromo-3-chloropropane (Whorton et al., 1979) and aromatic amines, respectively (Neumann, 2010). At present, very little is known about how xenobiotics generate oxidative stress or damage DNA in spermatozoa. Information concerning the relevant metabolizing enzymes and metabolic pathways in the testes and epididymides is also largely lacking. The chemical industry and its regulators consider them safe at the concentrations they are routinely used. However, the collective or combined damaging effect of so many environmental xenobiotics albeit ‘at safe concentrations’ is unknown and should be of concern.
The routine chemical insults experienced by all cell types, including spermatozoa, from endogenous and exogenous reactive chemicals or their metabolites, comprise three distinct chemical classes: radicals, ionic species and neutral but otherwise potentially highly reactive molecules. There are four recognized groups of such species (Fig. 2) designated by their reactive heteroatom: reactive oxygen/nitrogen/sulphur/halogen (chlorine or bromine) Species (Halliwell and Whitman, 2004). ROS are the most studied group and are commonly, but often incorrectly, used to mean all classes of reactive species. Last but not least are the environmental stressors such as heat and electromagnetic radiation, which are also well known to enhance mitochondrial ROS generation by human spermatozoa, decreasing the motility and vitality of these cells, while stimulating DNA base adduct formation and, ultimately DNA fragmentation (De Iulii et al., 2009a; Hourcade et al., 2010).

Given the plethora of such diverse attacking molecules, the type of damage sustained by DNA has to be equally diverse. The most recognized types of oxidative sperm DNA damage are: (i) single and double DNA strand breaks, (ii) the loss of a base to create an abasic site, (iii) the chemical modification of a base by, for example, oxidation or alkylation, (iv) inter- or intra-strand DNA cross linkage and (v) DNA-protein cross-links.

Equally important is the fact that oxidative stress originating from endogenous and exogenous stressors may be significantly augmented by a decline in local antioxidant protection, particularly during epididymal maturation. For example, low levels of seminal small molecule antioxidants such as vitamin C (Song et al., 2006) have been powerfully associated with semen quality, and strong correlations between antioxidant enzymes and oxidative DNA damage were recently reported in transgenic animals lacking a key epididymal enzyme, glutathione peroxidase 5 (GPx5) (Chabory et al., 2009).

Fortunately, there is a second line of defence against oxidative stress since mammalian oocytes have evolved complex mechanisms to identify DNA damage and activate the required response to maintain genomic integrity. These mechanisms include DNA damage detection, DNA repair, cell cycle arrest and apoptosis, which operate together to protect the embryo from DNA damage originating in either of the parental gametes. Moreover, since 90% of DNA is composed of introns, the probability that DNA damage will occur in the protein-coding regions or exons is relatively low, though clearly damage to intragenic regions can also contribute to pathogenic processes. These extenuating factors have been reflected in a number of studies where successful fertilization or establishment of pregnancy has been demonstrated using DNA-damaged spermatozoa obtained from patients undergoing IVF-ICSI treatment (Gandini et al., 2004). Nonetheless, the remarkable capacity of the oocyte for DNA repair may substantially differ between individuals and would depend on factors such as the type and extent of DNA damage as well as the age and quality of oocytes. Under normal circumstances, the oocyte is expected to repair low-level sperm DNA damage (Matsuda et al., 1989; Genesca et al., 1992) but when the damage is severe or the repair mechanisms are defective, as may occur with ageing, then the consequences may include impaired embryonic development (Hwang et al., 1997), pregnancy loss, birth defects or morbidity in later life (Ji et al., 1997; Sun et al., 1997). During natural conception, IUI or routine IVF, oxidative damage to the sperm plasma membrane might be expected to block fertilization, preventing the damaged paternal DNA from creating an embryo. However, during IVF-ICSI, this natural barrier to fertilization is lost, and sperm containing damaged DNA can still achieve fertilization following microinjection (Twigg et al., 1998). While many of these embryos will ultimately fail at the blastocyst or early embryonic stage, there is the potential for a child to be born with paternally derived DNA damage. The true consequences of this are unknown, but it has been suggested to include the initiation of genetic defects and other morbidities including childhood cancer (Aitken and Krausz, 2001; Aitken et al., 2003a).

In this context, it may be important that the results of a recent survey of more than 15 000 children born following assisted conception in France revealed significant increases in the incidences of major congenital malformations, Beckwith–Wiedemann syndrome and retinoblastoma (Viot et al., 2010).

Sperm DNA damage must therefore be regarded as a potential risk factor for the development of normal human embryos. Therefore the diagnosis and clinical management of sperm oxidative stress and DNA damage should be addressed in the name of ‘best practice’.

**Diagnosis of sperm oxidative stress and DNA fragmentation: association with clinical outcome**

The spermatozoon’s most important single function is to transport and deliver its precious DNA cargo, intact, to the oocyte. While routine semen analyses provide some insights concerning the number and general health of spermatozoa in infertile men, it gives little information about the overall integrity of the genomic DNA load they carry. One important question facing fertility researchers and clinicians is whether to include assays measuring oxidative stress and sperm DNA damage as part of the routine semen analysis for patients. This is mainly because the studies that have attempted to establish a relationship between the results of such assays and various clinical outcomes are generally considered inadequate because they are small, poorly designed and often lack control for female factors (Collins et al., 2008; Zini and Sigman, 2009). Nevertheless, there is general agreement that these assays link high levels of DNA damage with lower rates of natural conception or IUI/IVF success (Zini and Libman, 2006; Evenson and Wixon, 2008). Such studies, including a recent meta-analysis, also associate DNA damage with higher rates of pregnancy loss after IVF and IVF-ICSI treatments (Zini and Libman, 2006; Zini and Sigman, 2009).

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<tr>
<th>Endogenous Species</th>
<th>Reactive Oxygen Species</th>
<th>Reactive Nitrogen Species</th>
<th>Reactive Sulphur Species</th>
<th>Reactive Halogen Species</th>
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<tr>
<td>Radicals</td>
<td>OH·, LOO·</td>
<td>NO·, NO₂⁻</td>
<td>RS⁻</td>
<td>Cl⁻</td>
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<tr>
<td>Ionic</td>
<td>ONOO⁻</td>
<td>NO·, NO₂⁻</td>
<td>RS⁻</td>
<td>ClO₂⁻, BrO⁻</td>
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<td>Neutral but reactive</td>
<td>H₂O₂, O₂</td>
<td>N₂O₅, N₂O₄</td>
<td>H₂S</td>
<td>NO₂Cl, Cl⁻</td>
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**Figure 2** Examples of endogenous reactive molecular species often referred to as ROS.
Further support for the inclusion of these assays in the assessment of male infertility comes from a recent large study where over 60% of men attending fertility clinics were diagnosed with elevated levels of sperm DNA damage, with ~30% being severe (Cohen-Bacrie et al., 2009). These results suggest that a large number of patients attending IVF clinics are at a higher risk of IUI/IVF failure or miscarriage. This should be of particular concern to couples characterized by advanced maternal age or where oocyte quality is likely to be a particular issue.

As mentioned previously, the types of sperm DNA damage inflicted on sperm cells is varied. Accordingly many assays have been developed to detect and measure particular types of DNA damage (Agarwal and Allamaneni, 2005). Interestingly, a majority of these assays show a strong correlation with each other (Chohan et al., 2006; Santiso et al., 2010). The two most frequently employed assays are SCSA and TUNEL. SCSA measures the susceptibility of sperm DNA to acid hydrolysis using flow cytometry and is therefore generally considered as an indirect method of assessing DNA integrity. The technique has been extensively standardized with a defined threshold indicating fertility potential, albeit more probabilistic than deterministic (Evenson et al., 1980, 1999; Evenson and Wixon, 2008). TUNEL is a direct assay measuring actual DNA strand breaks (Sun et al., 1997; Henkel et al., 2010) and was recently optimized to increase its sensitivity in analyzing DNA fragmentation in human spermatozoa (Mitchell et al., 2010). A less common clinical test for oxidative DNA damage is the measurement of DNA oxidation adducts such as 8-OHdG by high-performance liquid chromatography or flow cytometry. In a recent publication, DNA fragmentation (measured by TUNEL) and oxidative DNA damage (measured by 8-OHdG levels) were found to be highly correlated (Aitken et al., 2010). This study suggested a diagnostic threshold of around 40% positive cells for both TUNEL and 8-OHdG formation in unfractionated sperm suspensions and around 25% following Percoll centrifugation.

Alternative strategies that might be used to detect oxidative stress in patients’ spermatozoa include the measurement of ROS employing, for example, luminol-dependent chemiluminescence (Aitken et al., 1991) or flow cytometry, following prior loading of the cells with dihydroethidium (De Luijis et al., 2006) or MitoSox Red™ (Koppers et al., 2008). The major problem with these assays is that they have to be conducted on freshly prepared cells because, by their very nature, ROS are short-lived. In addition, the measurement of ROS does not take account of the relative ability of the spermatozoa and reproductive tract fluids to scavenge these toxic metabolites. So, while the elevated generation of ROS is clearly correlated with male infertility, it is only one part of the redox equation. Measurement of the products of lipid peroxidation such as 4-hydroxyalkenals or malondialdehyde may more accurately reflect the net oxidative stress experienced by spermatozoa in their life history, although the relative hydrophilicity of these metabolites means that they do not remain permanently associated with the sperm plasma membrane. In light of these considerations, the measurement of 8-OHdG has much to commend it as a robust measure of oxidative stress that can still be used following fixation of the spermatozoa with paraformaldehyde and storage in glycinine buffer for at least 1 week (Aitken et al., 2010).

The development of novel methods and optimized thresholds for diagnosing oxidative DNA damage in human spermatozoa should assist in the clinical management of this pathology. Diagnosis of sperm oxidative stress and DNA damage will steer the clinicians and patients towards the best assisted reproductive technique to use. For example, the use of repeated IUI procedures and, to some degree IVF, may be averted. Patients may be urged to consider ICSI immediately, thus avoiding unnecessary delays, prolonged emotional trauma and extra costs. Patients with severe sperm DNA damage considering the use of assisted reproduction technique (ART) may be informed about the potential risks of miscarriage and childhood disease, thus creating much needed awareness and objectivity prior to family planning. These patients, particularly those exhibiting advanced maternal age or where the quality of oocyte may be compromised, could be advised to consider the use of high magnification ICSI or ICSI with testicular sperm. Both techniques look promising with studies reporting significant improvements in pregnancy outcome over conventional ICSI (Greco et al., 2005c; Antinori et al., 2008). The scientific premise here is that the sperm cells selected for ICSI by both techniques have substantially less DNA damage. Additionally, the patients may be urged to consider antioxidant therapy before undergoing ART, since antioxidant supplementation is generally associated with reduced levels of DNA damage or improved fertility potential (Tremellen, 2008).

In order to bridge the gap between the cutting edge of science and clinical practice in male infertility, future studies should be designed with adequate power so that a more precise association of sperm DNA damage with a range of clinical outcomes may be established. In such studies, the results obtained from a combination of assays measuring DNA damage such as 8-OHdG and SCSA and/or TUNEL in raw and processed samples should provide a more predictive insight into probable clinical outcomes. However, it is unrealistic or doubtful that any type of study will yield a highly defined dichotomous threshold, though a probabilistic one is still valuable.

**Pharmacological management of sperm oxidative stress with natural antioxidants**

Infertile men often exhibit elevated levels of ROS and/or diminished antioxidant capacity within their seminal plasma and spermatozoa (Fuji et al., 2003). Since sperm oxidative stress and DNA damage are recognized as significant factors in male infertility and achieving a healthy pregnancy, there is a clear rationale behind antioxidant treatment for infertile men. Spermatozoa are particularly vulnerable to oxidative stress not only because of their high polysaturated fatty acid content but also because of inherent deficiencies in intracellular antioxidant enzyme protection and a limited capacity for DNA repair. Fortunately, the reproductive tract, including the epididymal and seminal plasmas, contains a powerful array of enzymatic and non-enzymatic antioxidant molecules that act in concert to protect spermatzoa against a barrage of toxic oxygen metabolites. The scavenger enzymes superoxide dismutase, catalase and GPxs in semen are part of the first line defence against ROS. A recent example of the importance of such enzymes was afforded by deletion of GPx5 in male mice, which was found to generate a state of oxidative stress that influenced the incidence of miscarriage and birth defects in mated wild-type female mice, thus demonstrating the protection that this enzyme normally affords (Chabory et al., 2009).
Equally important, first line defence antioxidant molecules comprise a host of low molecular mass ROS scavengers such as vitamin C, E and many other naturally occurring antioxidants. Not surprisingly, several observational studies, involving semen samples obtained from subfertile/infertile men, show low concentrations of these molecules relative to that in semen samples obtained from fertile men (Fraga et al., 1996; Lewis et al., 1997; Balercia et al., 2000).

Based on the weight of such scientific evidence, numerous clinical studies have been carried out to establish the beneficial effects of oral antioxidants in improving sperm health and thus improve fertility. A search of MEDLINE and a survey of the relevant published literature reviews revealed some 65 such studies mostly conducted over the last two decades. In all, approximately a dozen antioxidants have been evaluated clinically either individually or in combination. However, most trials are small in size and differ in the target population selected as well as the type, dose and duration of antioxidant therapy. Several reviews of clinical studies addressing the effect of oral antioxidants on male infertility have been published recently (Lanzafame et al., 2009; Zini et al., 2009; Ross et al., 2010). The most recent review by Ross et al. (2010) gave a detailed account of some 17 studies selected strictly on the basis of randomization, with unselected infertile men as the target population taking oral antioxidants. The review reaches some important conclusions. Of the 17 studies, 13 reported improvement in at least one semen variable, following a varied regimen of oral antioxidant therapy. The review also found that 6 of 10 studies reported improved pregnancy rates and all of the studies (7 in total) showed a marked reduction of oxidative stress and/or DNA damage.

In the following analysis, we selected only the trials that assessed the effect of oral antioxidants against a measure of sperm oxidative stress or DNA damage. Of the 65 published trials, 23 studies report adequate data and were chosen for further analysis. Three were then deleted due to data clarity issues or difficulty in data interpretation. All studies reported their results in terms of semen variables or secondary outcomes such as fertilization rates or pregnancy, though the studies are small, heterogeneous and not amenable to meta-analysis. Here, we summarize the results, and Table I lists the study characteristics and results reported in the 20 trials incorporated into our analysis. All studies except one were single centre studies. Of the 20 studies, 11 were placebo-controlled, 13/20 were randomized and 13/20 had no follow-up. Overall, 19/20 studies showed a significant reduction of oxidative stress or DNA damage after oral antioxidant treatment. Among semen variables, motility was improved in 10 of 16 studies, but 100% of the studies (or 7/7) targeting asthenospermic patients showed a significant improvement in motility. There was no effect of antioxidants on sperm morphology and only three studies reported positive effects on concentration. A total of 10 studies provided secondary measures such as fertilization or pregnancy rates, with six reporting a significant improvement.

During the preparation of this manuscript, a meta-analysis of the impact of antioxidant therapy on male infertility was published, which reached the same generally positive conclusion, that supplementation significantly improves pregnancy and live birth rates in subfertile couples who used ART (Showell et al., 2011). The analysis presented by these authors is extremely detailed and readers are encouraged to consult this review for a breakdown of the studies that have been conducted in this area. It is clear from this extensive analysis that no perfect trials have been conducted to date, that involve the careful selection of patients with evidence of oxidative stress in their germ line, an adequate double-blind, crossover, randomized study design and a clinically relevant end-point, specifically pregnancy. These authors also emphasized that more research is needed to determine the optimal composition of the antioxidant formulations used to treat subfertile males and emphasized the importance of including DNA damage as one of the assessment criteria. The properties of some of the antioxidants assessed in these trials are presented subsequently.

The antioxidant efficacy of vitamin C alone on sperm oxidative stress was first demonstrated by Fraga et al. (1996) in a small number of smokers almost two decades ago. No other studies to confirm this finding or to establish its effect on pregnancy have since been attempted, although three other studies support the beneficial effects of vitamin C on various semen parameters (Dawson et al., 1987, 1982; Akmal et al., 2006). Vitamin E, on the other hand, was found to effectively reduce ROS concentration and improve fertilization or pregnancy rates in two studies. In contrast, two other trials with vitamin E reported no effect on any of the outcome variables (Giovenco et al., 1987; Molianen and Hovatta, 1995).

The positive effects of zinc on semen parameters have been known for some time and documented in at least five clinical studies (Tikkwal et al., 1987; Kynaston et al., 1988; Omu et al., 1998; Wong et al., 2002; Deng et al., 2005). Recently, a study by Omu et al. (2008) showed the antioxidant efficacy of zinc in reducing several measures of oxidative stress and improving motility in asthenospermic patients (Omu et al., 2008). Unfortunately, none of the studies measured secondary outcomes; so the effect of zinc on pregnancy rates remains unknown. L-Carnitine (LC) and acetyl-L-carnitine alone or in combination with each other are probably the most studied antioxidants in male infertility, with at least 17 trials documenting their effect (Moncada et al., 1992; Costa et al., 1994; Vitali et al., 1995; Vicari and Calogero, 2001; Vicari et al., 2002; Lenzi et al., 2003, 2004; Cavallini et al., 2004; Shang et al., 2004; Balercia et al., 2005; De Rosa et al., 2005; Garolla et al., 2005; Khademi et al., 2005; Li et al., 2005; Sigman et al., 2006). Of these five studies used a measure of sperm oxidative stress to assess the treatment effect, with four showing a significant reduction of oxidative stress/DNA damage and improvement in motility. Of the 12 other studies, 10 showed improvement in at least one semen variable, mostly motility. In 10 studies, there was a follow up of pregnancy outcome, with 4 showing improved rates but 6 reporting no significant effect. A meta-analysis of the results of some of the studies with carnitines strongly supports their role in increasing motility and pregnancy for infertile men (Zhou et al., 2007). A single study of N-acetyl-L-cysteine (NAC) published recently a reduction of oxidative stress and an improvement in sperm motility but no effect on pregnancy was observed (Ciftci et al., 2009). A larger trial of NAC supported the improvement in motility but also reported an increase in sperm concentration (Safarinejad and Safarinejad, 2009).

With the early observation that semen parameters could be improved by both hydrophilic and lipophilic antioxidants, much attention has focused on studying the combined effects of these agents in male infertility. At least 25 studies report various combinations in different populations of infertile men but only a dozen use a measure of sperm oxidative stress (Table I). Vitamin E, a well-known lipophilic antioxidant has been most widely used in combination with a variety of hydrophilic antioxidants such as vitamin C, selenium and zinc.
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<th>Author</th>
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<th>Morph</th>
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<td>Fraga et al. (1991)</td>
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<td>ND</td>
<td></td>
<td>↑, 8-OHdG</td>
<td>ND Smokers, controlled environment</td>
<td>10</td>
<td>NA</td>
<td>5–250 mg</td>
<td>15 weeks</td>
<td>Depletion/repletion</td>
</tr>
<tr>
<td>Vitamin E</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Suleiman et al. (1996)</td>
<td>ND</td>
<td>60/11</td>
<td>ND</td>
<td></td>
<td>↑, MDA</td>
<td>9 versus 0 live birth Asthenospermic (motility &lt;40%)</td>
<td>52</td>
<td>35</td>
<td>3 × 100 mg</td>
<td>6 months</td>
<td></td>
</tr>
<tr>
<td>Geva et al. (1996)</td>
<td>ND</td>
<td>ND</td>
<td>NE</td>
<td></td>
<td>↑, MDA</td>
<td>Fert. rate 19.3 → 29.1 Fertile normospermic with low FR</td>
<td>15</td>
<td>NA</td>
<td>200 mg</td>
<td>3 months</td>
<td>Prospective</td>
</tr>
<tr>
<td>Zinc</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Omu et al. (2008)</td>
<td>NE</td>
<td>↑</td>
<td>NE</td>
<td></td>
<td>↑, MDA, TAC, DFI</td>
<td>Asthenozoospermia ≥ 40% immotile</td>
<td>11</td>
<td>8</td>
<td>2 × 200 mg</td>
<td>3 months</td>
<td></td>
</tr>
<tr>
<td>L-Carnitine</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Balercia et al. (2005)</td>
<td>NS</td>
<td>↑</td>
<td>ND</td>
<td></td>
<td>↑, TOSC</td>
<td>Two pregnancy versus three placebo Idiopathic asthenozoospermia</td>
<td>15</td>
<td>15</td>
<td>3 g</td>
<td>6 months</td>
<td>Detailed motility</td>
</tr>
<tr>
<td>Acetyl-L-carnitine</td>
<td></td>
<td></td>
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<tr>
<td>Balercia et al. (2005)</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td></td>
<td>↑, TOSC</td>
<td>Three pregnancy versus three placebo Idiopathic asthenozoospermia</td>
<td>15</td>
<td>15</td>
<td>3 g</td>
<td>6 months</td>
<td>Detailed motility</td>
</tr>
<tr>
<td>Astaxantinhe</td>
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<tr>
<td>Comhaire et al. (2005)</td>
<td>NS</td>
<td>NS</td>
<td>NE</td>
<td></td>
<td>↑, ROS counts</td>
<td>Pregnancy (54.5 versus 10.5%)</td>
<td>11</td>
<td>19</td>
<td>16 mg</td>
<td>3 months</td>
<td>IUI</td>
</tr>
<tr>
<td>N-acetyl-L-cysteine</td>
<td></td>
<td></td>
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<tr>
<td>Ciftci et al. (2009)</td>
<td>NE</td>
<td>↑</td>
<td>NE</td>
<td></td>
<td>↑, OSI</td>
<td>Idiopathic with normal sperm parameters</td>
<td>60</td>
<td>60</td>
<td>600 mg</td>
<td>3 months</td>
<td>Also improvement in volume/viscosity</td>
</tr>
<tr>
<td>Vitamins C and E</td>
<td></td>
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<tr>
<td>Greco et al. (2005a)</td>
<td>NS</td>
<td>NE</td>
<td>NE</td>
<td></td>
<td>↑, TUNEL</td>
<td>Idiopathic non-smokers, DFI ≥ 15%</td>
<td>32</td>
<td>32</td>
<td>2 × 0.5 g each</td>
<td>2 months</td>
<td></td>
</tr>
<tr>
<td>Greco et al. (2005b)</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td></td>
<td>↑, TUNEL</td>
<td>2/29 versus 14/29 clinical pregnancies DFI ≥ 15%, ICSI patients (1 before and 1 after antioxidant treatment)</td>
<td>38</td>
<td>NA</td>
<td>2 × 0.5 g each</td>
<td>2 months</td>
<td>29 antioxidant responders</td>
</tr>
<tr>
<td>Vitamins E and Se</td>
<td></td>
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<tr>
<td>Keskus-Amma et al. (2003)</td>
<td>NE</td>
<td>↑</td>
<td>NE</td>
<td></td>
<td>↑, MDA</td>
<td>Infertile men</td>
<td>12</td>
<td>8</td>
<td>2 × 200 mg and 3 × 75 μg</td>
<td>3 months</td>
<td>Active control (vitamin B)</td>
</tr>
<tr>
<td>Vitamins E and Zn</td>
<td></td>
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<tr>
<td>Omu et al. (2008)</td>
<td>NE</td>
<td>↑</td>
<td>NE</td>
<td></td>
<td>↑, MDA, TAC, DFI</td>
<td>Asthenozoospermia ≥ 40% immotile</td>
<td>12</td>
<td>8</td>
<td>2 × 10 mg, 2 × 200 mg</td>
<td>3 months</td>
<td></td>
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<tr>
<td>Vitamins C, E and Zn</td>
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<tr>
<td>Omu et al. (2008)</td>
<td>NE</td>
<td>↑</td>
<td>NE</td>
<td></td>
<td>↑, MDA, TAC, DFI</td>
<td>Asthenozoospermia ≥ 40% immotile</td>
<td>14</td>
<td>8</td>
<td>2 × 5 mg, 2 × 10 mg, 2 × 200 mg</td>
<td>3 months</td>
<td></td>
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<tr>
<td>Vitamins C, E, glutathione</td>
<td></td>
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<tr>
<td>Study</td>
<td>Intervention</td>
<td>Participants</td>
<td>Outcomes</td>
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<tr>
<td>Kodama et al. (1997)</td>
<td>Vitamins C, E, Zn and b-carotene</td>
<td>Infertile men</td>
<td>8-OHdG ND 14 NA 200 mg, 200 mg, 400 mg 2 months</td>
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<tr>
<td>Ménézo et al. (2007)</td>
<td>Vitamins C, E, Zn, folate acid and garlic</td>
<td>2-failed IVF/ICSI cycles and DFI &gt; 15% 58 NA 400 mg, 400 mg, 1 μmol, 500 μmol, 18 mg 90 days Sperm decond. observed</td>
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<tr>
<td>Tremellen et al. (2007)</td>
<td>Vitamins C, E, Se, Zn, folic acid and garlic</td>
<td>38.5 versus 16% pregnancy rate at 13 weeks gestation IVF-ICSI patients TUNEL +ve &gt;25% and poor morphology or low motility 36 16 100 mg, 400 IU, 26 μg, 25 mg, 0.5 mg, 6 mg, 1 g 3 months Patent reports no TUNEL effect [125]</td>
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<tr>
<td>Tunc. et al. (2009)</td>
<td>Vitamins C, E, Se, Zn, folic acid and garlic</td>
<td>Men exhibiting oxidative stress 50 NA 100 mg, 400 IU, 26 μg, 25 mg, 0.5 mg, 6 mg, 1 g 3 months</td>
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<tr>
<td>Balercia et al. (2005)</td>
<td>Vitamins C, E, Se, Zn, folic acid and garlic</td>
<td>Five pregnancy versus three placebo Idiopathic asthenozoospermia 14 15 2 g and 1 g 6 months Detailed motility</td>
<td></td>
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</tbody>
</table>

C, concentration; DFI, DNA fragmentation index; M, motility; MDA, malondialdehyde; Morph, morphology; ND, not determined; NE, no effect; NS, not significant; 2^2 Outcomes: fertilization rate (FR) or pregnancy; OS, oxidative stress; Placb, Placebo; PVE, prostatovesiculoididymitis; TAC, total antioxidant capacity; WBC, white blood cell.
in addition to other miscellaneous combinations. Somewhat surprisingly, the results from the 12 combination studies mirror those observed with single antioxidants with no apparent synergistic effects on any of the outcome variables. Although, 11 of the 12 studies confirmed a significant reduction of sperm oxidative stress or DNA damage, only 5 of the 10 studies that measured motility showed improvement. None of the studies showed any significant improvement in morphology and only 2 of 10 studies showed an improvement in sperm concentration.

In summary, to date, almost every study that has measured the effect of antioxidants on sperm oxidative stress or DNA damage indicates a significant improvement. Additionally, the effect of antioxidants on motility appears to be compelling, particularly in asthenospermic patients. Such improvements in sperm motility are clinically and biologically important given the wealth of data linking the movement characteristics of human spermatozoa with the fertilizing potential of these cells in vivo and in vitro (Aitken, 2006). However, the results of the 20 studies summarized in Table I do not differentiate an impact upon normal embryo development. Patients with high levels of sperm oxidative stress and DNA damage affect male fertility and may logically important given the wealth of data linking the movement characteristics of human spermatozoa with the fertilizing potential of these cells in vivo and in vitro (Aitken, 2006). However, the results of the 20 studies summarized in Table I do not differentiate an appreciable advantage for lipophilic versus hydrophilic antioxidants nor reveal any additive or synergistic effects when combined together. However, it should be emphasized that a majority of these studies were small, heterogeneous and did not generate sufficient data to confirm whether the improvements gained by antioxidant therapy result in improved secondary outcomes.

Thus, the quest to identify novel antioxidants and combinations that are optimized for safety and efficacy is likely to continue. On theoretical grounds, an appropriate combination of antioxidants should be more effective than any single antioxidant since oxidative stress is a non-localized heterogeneous phenomenon. For example, vitamin C, carnitines, zinc and NAC are all highly hydrophilic molecules; conversely, vitamin E and carotenoids such as astaxanthin are highly lipophilic structures. Each of these naturally occurring antioxidants with their own unique pharmacodynamic profile for the male reproductive tract is likely to neutralize at least some of the nearby ROS, thus collectively transpiring to a more effective management of sperm oxidative stress. However, with a wide selection of antioxidants to choose from, designing an optimum combination will be a challenge. In developing optimized combinations, particular attention should be paid to the doses and number of ingredients used. Large doses of antioxidants should be avoided due to specific reactions resulting in possible negative effects. For example, high doses of vitamin C is reported to reduce the interchain disulphide bridges in protamines opening the cysteine net and subsequently promoting DNA decondensation in spermatozoa (Donnelly et al., 1999; Ménézo et al., 2007; Giustarini et al., 2008). Selenium at higher doses significantly reduces the number of motile spermatozoa in fertile men possibly through modifying thyroid hormone metabolism (Hawkes and Turek, 2001). The doses of antioxidants become even more of a consideration when large numbers of compounds are packed into a particular formulation as risks of depleting the essential physiological levels of ROS becomes real, potentially leading to ‘reductive stress’ and the impairment of normal sperm function (Lipinski, 2002).

**Summary and conclusion**

Sperm oxidative stress and DNA damage affect male fertility and may impact upon normal embryo development. Patients with high levels of sperm DNA damage undergoing ART treatment are at a greater risk of fertilization failure, pregnancy loss or not achieving a healthy progeny. These risks escalate further if the oocyte’s DNA repair mechanisms are compromised. Under the current status quo, sperm DNA damage remains largely undiagnosed and untreated even though one in three men attending IVF clinics exhibit severely elevated levels. From our review of the evidence in 20 clinical studies, there is little doubt over the effectiveness of oral antioxidants to reduce sperm oxidative stress and increase motility in asthenospermic patients, but it is as yet unclear whether this improvement translates to higher full-term pregnancy rates. It is therefore imperative that future clinical trials with antioxidants target patients with moderate to severe sperm oxidative stress and demonstrate improvement in as many measurable secondary outcomes as possible but, more critically, pregnancy and miscarriage rates. The trials should be large, carefully designed, if possible multi-centred, double-blind, randomized, cross-over with strict selection criteria applied to both genders.

**Authors’ roles**

P.G. conceived and drafted the article, which was then edited and revised by R.J.A. Both authors approved the final version of this article.

**Conflict of interest**

P.G. is the Managing Director of CellOxess LLC, which has a commercial interest in the detection and resolution of oxidative stress.

**References**


Antioxidants in the treatment of male infertility


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