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Oxidative stress and male infertility—a clinical perspective

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Oxidative stress occurs when the production of potentially destructive reactive oxygen species (ROS) exceeds the bodies own natural antioxidant defenses, resulting in cellular damage. Oxidative stress is a common pathology seen in approximately half of all infertile men. ROS, defined as including oxygen ions, free radicals and peroxides are generated by sperm and seminal leukocytes within semen and produce infertility by two key mechanisms. First, they damage the sperm membrane, decreasing sperm motility and its ability to fuse with the oocyte. Second, ROS can alter the sperm DNA, resulting in the passage of defective paternal DNA on to the conceptus. This review will provide an overview of oxidative biochemistry related to sperm health and will identify which men are most at risk of oxidative infertility. Finally, the review will outline methods available for diagnosing oxidative stress and the various treatments available.

Keywords: oxidative stress; sperm; male infertility; antioxidant; treatment options

Introduction

Male factor infertility accounts for up to half of all cases of infertility and affects one man in 20 in the general population (McLachlan and de Kretser, 2001). Evidence now suggests that reactive oxygen species (ROS)-mediated damage to sperm is a significant contributing pathology in 30–80% of cases (Iwasaki and Gagnon, 1992; Zini *et al*., 1993; Ochsendorf, 1994; Shekarriz *et al*., 1995a, b; Agarwal *et al*., 2006a). ROS, defined as including oxygen ions, free radicals and peroxides, cause infertility by two principal mechanisms. First, ROS damage the sperm membrane which in turn reduces the sperm's motility and ability to fuse with the oocyte. Secondly, ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo. Despite the common association between compromised sperm quality and oxidative damage, men are rarely screened for oxidative stress nor treated for this condition. Instead they are usually offered 'mechanical' treatments such as intracytoplasmic sperm injection (IVF-ICSI) or intrauterine insemination (IUI). This is less than optimal as oxidative damage to sperm DNA is not directly ameliorated by either IVF-ICSI or IUI treatment. In addition, direct treatment of oxidative stress may allow for natural conception, thereby conserving scarce medical resources. This review will provide an overview of who is at risk of oxidative stress, the mechanisms by which oxidative stress produces infertility and the methods available for its diagnosis and treatment.

Overview of oxidative stress biochemistry

ROS are products of normal cellular metabolism. Most of the body's energy is produced by the enzymatically controlled reaction of oxygen with hydrogen in oxidative phosphorylation occurring within the mitochondria during oxidative metabolism. During this enzymatic reduction of oxygen to produce energy, free radicals are formed (Valko *et al*., 2007). A free radical is defined as an oxygen molecule containing one or more unpaired electrons in atomic or molecular orbitals. The addition of one electron to dioxygen (O_2) forms the superoxide anion radical ($O_2^{\bullet -}$), the primary form of ROS. This superoxide anion can then be directly or indirectly (enzymatic, metal catalyzed) converted to secondary ROS such as the hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$) or hydrogen peroxide (H_2O_2). The terms free radical and ROS are commonly used in an interchangeable manner, despite the fact that not all ROS are free radicals (Cheeseman and Slater, 1993). For example, hydrogen peroxide (H_2O_2) is considered a ROS but it is not a free radical since it does not contain unpaired electrons. In addition, there is a sub-class of free radicals derived from nitrogen which includes nitrous oxide, peroxynitrite, nitroxyl anion and peroxynitrous acid. Free radicals seek to participate in chemical reactions that relieve them of their unpaired electron, resulting in the oxidation of lipids in membranes, amino acids in proteins and carbohydrates within nucleic acids (Ochsendorf, 1999).

Within semen there are two principal sources of production of free radicals; leukocytes and sperm. The vast majority of semen specimens contain leukocytes, with neutrophils being the predominant leukocyte type (Aitken *et al*., 1994; Aitken and Baker, 1995). As the production of ROS is one of the principal mechanisms by which neutrophils destroy pathogens, it is not surprising that seminal leukocytes have the potential to cause oxidative stress. However, a link between the presence of leukocytes in semen and male oxidative infertility is still under debate (Wolff, 1995). Several researchers have reported a positive correlation between seminal leukocyte numbers and ROS production (Aitken *et al*., 1994; Whittington *et al*., 1999; Sharma *et al*., 2001). However, other studies have failed to find a significant difference in seminal leukocyte concentration between fertile and infertile men (Christiansen *et al*., 1991; Tomlinson *et al*., 1993; Aitken and Baker, 1995; Rodin *et al*., 2003), and the activation state of leukocytes must also play an important role in determining final ROS output. This is supported by the observation of a positive correlation between seminal ROS production and pro-inflammatory seminal plasma cytokines such as interleukin IL-6 (Camejo *et al*., 2001; Nandipati *et al*., 2005), IL-8 (Rajasekaran *et al*., 1995; Martinez *et al*., 2007) and tumour necrosis factor TNFα (Sanocka *et al*., 2003; Martinez *et al*., 2007).

Every human ejaculate contains leukocytes which make the quantification of spermatozoa-specific ROS production more complex. However, sperm isolation techniques have been used to confirm that spermatozoa themselves are responsible for some ROS generation, not just contaminating seminal leukocytes (Baker *et al*., 2003). Separation of sperm from seminal leukocytes using density-gradient centrifugation has shown the 'sperm fraction' to produce significant ROS. As this fraction may still contain a very low number of leukocytes, experiments have been conducted where leukocytes are further depleted using magnetic beads coated with leukocyte-specific CD45 antibodies (Aitken *et al*., 1996). After removing all detectable leukocyte contamination, ROS production can still be recorded, confirming the ability of sperm to generate ROS. The relative importance of sperm and leukocyte production of ROS varies between individuals but can be estimated using the leukocyte specific activator, *N*-formyl-methionine-leucine-phenylalanine (FMLP).

The ability of sperm to produce ROS inversely correlates with their maturational state. During spermatogenesis there is a loss of cytoplasm to allow the sperm to form its condensed, elongated form. Immature teratozoospermic sperm are often characterized by the presence of excess cytoplasmic residues in the mid-piece. These residues are rich in the enzyme glucose-6-phosphate dehydrogenase, an enzyme which controls the rate of glucose flux and intracellular production of β-nicotinamide adenine dinucleotide phosphate (NADPH) through the hexose monophosphate shunt. NADPH is used to fuel the generation of ROS via NADPH oxidase located within the sperm membrane (Gomez *et al*., 1996; Fisher and Aitken, 1997; Said *et al*., 2005). As a result, teratozoospermic sperm produce increased amounts of ROS compared with morphologically normal sperm.

The existence of NADPH oxidase activity within sperm was questioned when addition of NADPH was unable to elicit any production of the superoxide anion measured by electron paramagnetic resonance spectroscopy, a very sensitive and specific assay for the superoxide anion (Richer and Ford, 2001). However, since then the presence of a calcium-dependant NADPH oxidase called NOX 5 has been confirmed within sperm (Banfi *et al*., 2001; Armstrong *et al*., 2002; Sabeur and Ball, 2007). This sperm-specific NADPH oxidase (NOX 5) is reported to be quite distinct from leukocyte NADPH oxidase, with NOX 5 activity not being controlled by protein kinase C as occurs in the leukocyte (Armstrong *et al*., 2002). Whether NOX 5 is over expressed in spermatozoa of patients exhibiting infertility associated with oxidative stress is presently unknown.

The relative importance of leukocytes and sperm in the aetiology of oxidative stress is currently under debate. The rate of production of ROS by leukocytes is reported to be 1000 times higher than that of spermatozoa at capacitation (Plante *et al*., 1994), making leukocytes the likely dominant producer of seminal ROS. When seminal ROS production is divided into that produced by the sperm themselves (intrinsic ROS) and that made by the leukocytes (extrinsic), an interesting observation is seen (Henkel *et al*., 2005). While both intrinsic and extrinsic ROS production is negatively correlated with sperm DNA integrity, the relationship is significantly stronger for intrinsic ROS production. This suggests that while leukocytes produce more ROS than sperm on a per cell basis, the close proximity between intrinsic ROS production and sperm DNA makes intrinsic ROS production a more important variable in terms of fertility potential.

The human body has developed several antioxidant strategies to protect itself from ROS damage. This allows for normal oxidative metabolism to occur without damaging the cells, while still allowing for normal ROS-mediated cellular responses such as destruction of infectious pathogens and intracellular signalling (Valko *et al*., 2007). Oxidative stress occurs when the production of ROS overwhelms the antioxidant defense mechanisms leading to cellular damage. Seminal plasma and sperm themselves are well endowed with an array of protective antioxidants (Fujii *et al*., 2003; Garrido *et al*., 2004a). Superoxide dismutase (SOD) and catalase are enzymatic antioxidants which inactivate the superoxide anion ($O_2^{\bullet -}$) and peroxide (H_2O_2) radicals by converting them into water and oxygen. SOD is present within both sperm and seminal plasma (Mennella and Jones, 1980; Zini *et al*., 1993). The addition of SOD to sperm in culture has been confirmed to protect them from oxidative attack (Kobayashi *et al*., 1991). While some investigators have reported minor reductions in seminal plasma SOD activity in infertile men (Alkan *et al*., 1997; Sanocka *et al*., 1997), many have not (Miesel *et al*., 1997; Zini *et al*., 2000; Hsieh *et al*., 2002). However, the majority of evidence does support a link between deficient seminal catalase activity and male infertility (Jeulin *et al*., 1989; Alkan *et al*., 1997; Miesel *et al*., 1997; Sanocka *et al*., 1997; Zini *et al*., 2000). Glutathione peroxidase (GPX) is the final member of the seminal enzymatic antioxidant triad. GPX consists of a family of antioxidants (GPX1-5) that are involved in the reduction of hydroperoxides using glutathione as an electron donor. The GPXs are located within the testis, prostate, seminal vesicles, vas deferens, epididymis, seminal plasma and spermatozoa themselves (reviewed by Vernet *et al*., 2004). GPX must play an important protective role against oxidative attack since its specific inhibition *in vitro* using mercaptosuccinate leads to a large increase in sperm lipid peroxidation (Twigg *et al*., 1998). Male factor infertility has been linked with a reduction in seminal plasma (Giannattasio *et al*., 2002) and spermatozoa (Garrido *et al*., 2004b) GPX activity,

further supporting an important role for this enzyme in male fertility. In addition, men exhibiting leukospermia-associated oxidative stress have been reported to have significantly reduced GPX activity within their spermatozoa (Therond *et al*., 1996). Finally, the continued activity of GPX depends on the regeneration of reduced glutathione by glutathione reductase (GTR). Selective inhibition of GTR reduces the availability of reduced glutathione for maintaining GPX activity, thereby exposing sperm to oxidative stress (Williams and Ford, 2004). The coordinated activity of GPX, GTR and glutathione clearly play a pivotal role in protecting sperm from oxidative attack.

The non-enzymatic antioxidants present within semen include ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione, amino acids (taurine, hypotaurine), albumin, carnitine, carotenoids, flavenoids, urate and prostasomes. These agents principally act by directly neutralizing free radical activity chemically. However, they also provide protection against free radical attack by two other mechanisms. Albumin can intercept free radicals by becoming oxidized itself, thereby sparing sperm from attack (Twigg *et al*., 1998). Alternatively, extracellular organelles (prostasomes) secreted by the prostate have been shown to fuse with leukocytes within semen and reduce their production of free radicals (Skibinski *et al*., 1992; Saez *et al*., 1998). A substantial number of researchers have reported a significant reduction in non-enzymatic antioxidant activity in seminal plasma of infertile compared with fertile men (Fraga *et al*., 1991; Fraga *et al*., 1996; Smith *et al*., 1996; Therond *et al*., 1996; Lewis *et al*., 1997; Gurbuz *et al*., 2003; Koca *et al*., 2003; Mostafa *et al*., 2006; Song *et al*., 2006).

Antioxidants contained within seminal plasma are obviously helpful for preventing sperm oxidative attack following ejaculation. However, during spermatogenesis and epididymal storage, the sperm are not in contact with seminal plasma antioxidants and must rely on epididymal/testicular antioxidants and their own intrinsic antioxidant capacity for protection. Sperm are therefore vulnerable to oxidative damage during epididymal transit, especially when there is epididymal inflammation such as male genital tract infection. In addition, testicular biopsies from men with varicocele-associated oxidative stress have shown an increase in oxidative DNA damage within spermatogonia and spermatocytes (Ishikawa *et al*., 2007). Therefore, while seminal plasma antioxidants may help minimize ejaculated sperm oxidative stress, they have no capacity to prevent oxidative damage initiated 'up stream' at the level of the testis and epididymis.

Seminal free radicals—friend or foe?

Sperm were the first type of cell reported to produce free radicals. In this pioneering report, MacLeod (1943) noted that incubation of sperm under conditions of high oxygen tension lead to a rapid loss of their motility. The addition of the antioxidant catalase to the medium preserved sperm motility, prompting MacLeod to suggest that sperm must produce hydrogen peroxide during normal oxidative metabolism. Since this publication, it has evolved that three inter-related mechanisms account for oxidative stress-mediated male infertility—impaired motility, impaired fertilization and oxidative DNA damage.

The underlying pathology behind free radicals ability to reduce sperm motility was first reported by Jones *et al*. (1979). They reported that ROS-induced peroxidation of the sperm membrane decreasing its flexibility and therefore tail motion. Sperm membranes are vulnerable to this type of damage as they contain large amounts of unsaturated fatty acids. Direct ROS damage to mitochondria, decreasing energy availability, may also impede sperm motility (de Lamirande and Gagnon, 1992; de Lamirande *et al*., 1997, 1998). By either mechanism, oxidative stress impairs sperm motility and will result in less sperm reaching the oocyte for fertilization (Whittington *et al*., 1999; Kao *et al*., 2007).

Low level production of free radicals by sperm plays a positive role in preparation for fertilization (capacitation). Hydrogen peroxide stimulates the acrosome reaction and sperm hyperactivation (de Lamirande and Gagnon, 1993), thereby assisting the sperm's transit through the cumulus and zona pellucida. Low concentrations of hydrogen peroxide also cause tyrosine phosphorylation, which augments sperm membrane binding to the zona pellucida ZP-3 protein (Aitken *et al*., 1995b), ultimately boosting sperm–oocyte fusion (Aitken *et al*., 1998). However, high levels of ROS production lead to peroxidation of the sperm acrosomal membrane and diminished acrosin activity (Zalata *et al*., 2004), and impaired sperm–oocyte fusion (Aitken *et al*., 1989; Ichikawa *et al*., 1999; Saleh *et al*., 2003a, b; Zorn *et al*., 2003a; Jedrzejczak *et al*., 2005).

Free radicals have the ability to directly damage sperm DNA by attacking the purine and pyrimidine bases and the deoxyribose backbone. Normally, sperm DNA is tightly packaged by protamines protecting it from free radical attack. However, infertile men often exhibit deficient protamination, leaving the sperm DNA particularly vulnerable to ROS attack (Oliva, 2006). Alternatively, free radicals can initiate apoptosis within the sperm, leading to caspase-mediated enzymatic degradation of the DNA (Kemal Duru *et al*., 2000; Wang *et al*., 2003; Moustafa *et al*., 2004; Villegas *et al*., 2005). Several investigators (Kodama *et al*., 1997; Aitken *et al*., 1998; Saleh *et al*., 2002b; Oger *et al*., 2003; Wang *et al*., 2003; Henkel *et al*., 2005; Kao *et al*., 2007) have now confirmed the link between oxidative stress and sperm DNA damage using various techniques such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), sperm chromatin structure assay (SCSA) and measurement of the byproduct of DNA oxidation, 8-hydroxydeoxyguanosine (8-OHdG). Furthermore, two groups have now correlated increased sperm oxidative DNA damage with poor blastocyst formation *in vitro* (Zorn *et al*., 2003a; Meseguer *et al*., 2006, 2007). Damaged paternal DNA is recognized to be a significant cause for poor blastocyst development (Seli *et al*., 2004). Finally, a large prospective study of 225 couples planning their first pregnancy found a strong inverse relationship between seminal 8-OHdG concentration and monthly natural fecundity (Loft *et al*., 2003).

During natural conception or routine IVF, oxidative damage to the sperm membrane will normally 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 significantly damaged DNA can still achieve fertilization following microinjection (Zorn *et al*., 2003a). While many of these embryos will ultimately fail at the blastocyst or early fetal stage, there is the potential for a child to be born with damaged paternal derived DNA. The consequences of this are as yet unknown but it has been suggested to include the initiation of genetic defects and childhood cancer (Aitken and Krausz, 2001; Aitken *et al*., 2003).

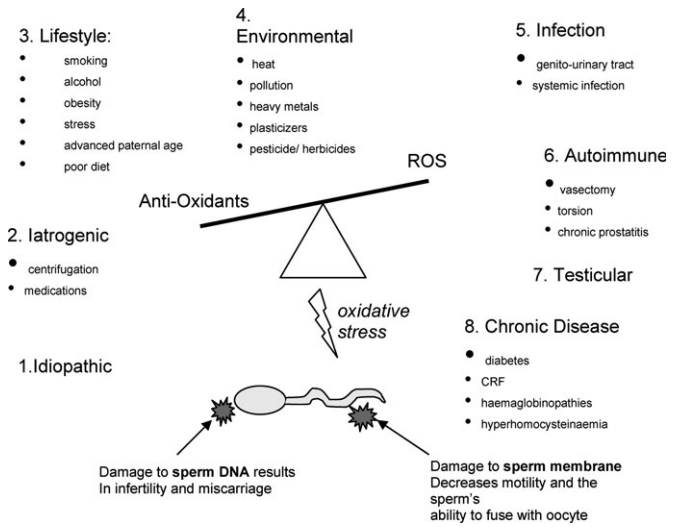

Figure 1: The oxidative stress balance.

Origins of oxidative stress

The origins of sperm oxidative stress are summarized in Fig. 1. While pathologies such as genitourinary tract infection and varicocele are well established causes of oxidative stress, others such as hyper-homocysteinaemia and diabetes are only now just becoming recognized as possible causes. It is hoped that this review will stimulate further research in these less well established potential causes of male oxidative infertility.

Idiopathic

Idiopathic male factor infertility has been linked with oxidative stress by several research groups. One of the principal causes of this association is the observation that morphologically abnormal sperm have an increased capacity to generate ROS, but also a reduced antioxidant capacity (Gomez *et al*., 1996; Garrido *et al*., 2004b; Said *et al.*, 2004; Said *et al*., 2005). As approximately one-third of infertile men exhibit teratozoospermia (Thonneau *et al*., 1991), it is not surprising that sperm oxidative stress is commonly identified in the idiopathic infertile male population. Even men with normozoospermic idiopathic infertility exhibit significantly higher seminal ROS production and lower antioxidant capacity than fertile men (Pasqualotto *et al*., 2001; Agarwal *et al*., 2006b), for as yet unknown reasons.

Iatrogenic

The use of assisted reproductive technologies (ART) has the potential to exacerbate sperm oxidative stress. During IVF and IUI treatment semen is centrifuged to separate sperm from seminal plasma. This exacerbates oxidative stress as centrifugation increases sperm ROS production many fold (Iwasaki and Gagnon, 1992; Shekarriz *et al*., 1995a, b), while removing sperm from protective antioxidants within seminal plasma (Potts *et al*., 2000a, b). In addition cryopreservation of sperm, another commonly used technique in ART, is associated with an increase in sperm oxidative stress (Watson, 2000).

Drugs such as the chemotherapy agent cyclophosphamide have been linked with sperm oxidative stress. Administration of cyclophosphamide to animals is reported to increase testicular malondialdehyde (MDA) levels and produce a fall in testicular catalase, implying the presence of oxidative stress (Das *et al*., 2002; Ghosh *et al*., 2002). Drugs such as aspirin and paracetamol (acetaminophen) can also produce oxidative stress by increasing cytochrome P450 activity, thereby boosting ROS generation (Agarwal and Said, 2005).

Lifestyle

Smoking results in a 48% increase in seminal leukocyte concentrations and a 107% increase in seminal ROS levels (Saleh *et al*., 2002a). Smokers have decreased levels of seminal plasma antioxidants such as Vitamin E (Fraga *et al*., 1996) and Vitamin C (Mostafa *et al*., 2006), placing their sperm at additional risk of oxidative damage. This has been confirmed by the finding of a significant increase in levels of 8-OHdG within smoker's seminal plasma (Fraga *et al*., 1996).

Dietary deficiencies have been linked with sperm oxidative damage by several research groups. The Age and Genetic Effects in Sperm (AGES) study examined the self-reported dietary intake of various antioxidants and nutrients (vitamins C and E, β-carotene, folate and zinc) in a group of 97 healthy non-smokers and correlated this with sperm quality (Eskenazi *et al*., 2005). This study did observe a significant correlation between vitamin C intake and sperm concentration and between vitamin E intake and total progressively motile sperm. This is also consistent with earlier reports of a significant link between seminal plasma vitamin E levels and an increase in percentage of motile sperm (Therond *et al*., 1996). However, the AGES study was unable to confirm a link between low intake of antioxidants and sperm DNA damage (Silver *et al*., 2005). This was surprising given that other researchers had linked low seminal plasma vitamin C levels with increased sperm DNA damage (Fraga *et al*., 1991; Song *et al*., 2006). It is possible that levels of individual antioxidants within seminal fluids may more accurately reflect biological effect than self-reported dietary intake as different food sources and preparation techniques can vastly modify antioxidant intake. Alternatively, differences in the populations studied may explain the discrepant results. Song *et al*. (2006) correlated sperm DNA damage with dietary antioxidant intake in infertile men, while Silver *et al*. (2005) and Fraga *et al*. (1991) examined this relationship in healthy presumed fertile patients. Fertile men with low levels of oxidative attack may not be as dependant on seminal antioxidants for protection of their sperm DNA integrity. Therefore, a dietary deficiency in antioxidants may not lead to sperm oxidative DNA damage in this fertile cohort.

Excessive alcohol consumption causes an increase in systemic oxidative stress as ethanol stimulates the production of ROS, while many alcohol abusers have diets deficient in protective antioxidants (Wu and Cederbaum, 2003; Koch *et al*., 2004). A study of 46 alcoholic men of reproductive age has suggested the presence of oxidative stress within the testicle by reporting a significant reduction in plasma testosterone, increase in serum lipid peroxidation byproducts and a drop in antioxidants (Maneesh *et al*., 2006). However, no study to date has directly examined the link between alcohol intake and sperm oxidative damage.

Extremes of exercise activity, at both ends of the spectrum, have been linked with oxidative stress. It is not surprising that high impact exercise is linked with oxidative stress since muscle aerobic metabolism creates a large amount of ROS (Peake *et al*.,

2007). In a rodent model, increasing levels of exercise are linked with a reduction in sperm count and motility and a corresponding increase in biochemical signs of testicular oxidative stress (Manna *et al*., 2004). Conversely, obesity produces oxidative stress as adipose tissue releases pro-inflammatory cytokines that increase leukocyte production of ROS (Singer and Granger, 2007). Furthermore, accumulation of adipose tissue within the groin region results in heating of the testicle which has been linked with oxidative stress and reduced sperm quality (Banks *et al*., 2005; Ishii *et al*., 2005; Perez-Crespo *et al*., 2007).

Psychological stress produces a reduction in semen quality; with the underlying mechanism previously felt to be related to a central impairment of gonadotrophin drive (Fenster *et al*., 1997). However, recent prospective studies have linked a period of psychological stress with a reduction in sperm quality mediated by an increase in seminal plasma ROS generation and a reduction in antioxidant protection (Eskiocak *et al*., 2005, 2006).

Several studies have reported that sperm DNA damage increases with advancing age in both fertile (Wyrobek *et al*., 2006) and infertile men (Singh *et al*., 2003; Moskovtsev *et al*., 2006). It is possible that an increase in oxidative sperm DNA damage is the underlying pathology. A large observational study has confirmed that systemic oxidative stress increases with age (Junqueira *et al*., 2004). Animal studies using the Brown Norway rat, an established model of male reproductive aging, confirm that sperm from older animals produce more free radicals than from young animals and have a reduced enzymatic antioxidant activity, resulting in an increase in ROS-mediated sperm DNA damage (Zubkova *et al*., 2005; Weir and Robaire, 2007).

Environmental

Phthalates are chemicals used as a plastics softener and are contained in a wide range of food packaging and personal care products. Exposure to phthalates can occur via dietary consumption, dermal absorption or inhalation and has been linked with impaired spermatogenesis and increased sperm DNA damage (Agarwal *et al*., 1985; Srivastava *et al*., 1990; Kasahara *et al*., 2002; Hauser *et al*., 2007). Oral administration of phalate esters to rats is reported to increase the generation of ROS within the testis and a concomitant decrease in antioxidant levels, culminating in impaired spermatogenesis (Lee *et al*., 2007).

Several environmental pollutants have been linked with testicular oxidative stress. Pesticides such as lindane (Chitra *et al*., 2001), methoxychlor (Latchoumycandane *et al*., 2002) and the herbicide dioxin-TCDD (Latchoumycandane *et al*., 2003) have all been linked with testicular oxidative stress in rodent models. The commonly used preservative sulfur dioxide has also been shown to produce testicular oxidative stress in laboratory animals (Meng and Bai, 2004). Air pollutants such as diesel particulate matter act as potent stimuli for leukocyte ROS generation (Gonzalez-Flecha, 2004; Alaghmand and Blough, 2007). While no study has directly linked airborne pollutants with testicular oxidative stress, it is possible that this oxidative insult is responsible for the increase in sperm DNA damage seen following periods of airborne pollution (Rubes *et al*., 2005).

Heavy metal exposure has been conclusively linked with sperm oxidative damage. Both cadmium and lead are linked with an increase in testicular oxidative stress (Hsu and Guo, 2002; Acharya *et al*., 2003) and a resultant increase in sperm DNA oxidation (Xu *et al*., 2003; Naha and Chowdhury, 2006). The increase in infertility and miscarriage observed in the partners of welders and battery/paint factory workers (Gennart *et al*., 1992; Bonde, 1993) may be due to oxidative damage to sperm DNA initiated by the inhalation of metal fumes.

Infection

Genitourinary tract infection

Up to 50% of men will experience prostatitis at some point in their lives, with prostatitis becoming chronic in 10% of men (Schaeffer, 2003). Bacteria responsible for prostate infection may originate from the urinary tract or can be sexually transmitted (Fraczek and Kurpisz, 2007; Fraczek *et al*., 2008). Typical non-sexually-transmitted pathogens include *Streptococci* (*S. viridans* and *S. pyogens*), coagulase-negative *Staphylococci* (*S. epidermidis*, *S. haemolyticus*), gram-negative bacteria (*E. coli*, *Proteus mirabilis*) and atypical mycoplasma strains (*Ureaplasma urealyticum*, *Mycoplasma hominis*). All of these pathogens will create an acute inflammatory response with an influx of leukocytes into the genital tract and a resulting increase in ROS production (Mazzilli *et al*., 1994; Depuydt *et al*., 1996; Ochsendorf, 1999; Potts *et al*., 2000a, b). Men prone to recurrent genitourinary tract infections, such as paraplegics, have been confirmed to have high degrees of sperm oxidative pathology (Padron *et al*., 1997; Brackett *et al*., 2008). Current or past *Chlamydia* infection has also been linked with an increase in oxidative damage to sperm (Segnini *et al*., 2003).

Viral infections may also initiate oxidative damage to sperm. The link between common viral pathogens such as cytomegalovirus, herpes simplex virus (HSV), Epstein-Barr virus and oxidative infertility has been examined by several groups. Only HSV appears to have a possible role in the initiation of oxidative damage to sperm. Herpes simplex DNA is found in 4–50% of infertile men's semen (Kapranos *et al*., 2003, Bezold *et al*., 2007), with IgM antibodies towards HSV being associated with a 10-fold increase in the rate of leukospermia (Krause *et al*., 2002, 2003). Given the well recognized link between leukospermia and seminal ROS levels, together with the observation of a reduction in sperm motility in men positive for seminal HSV DNA (Kapranos *et al*., 2003), it is likely that HSV is a viral pathogen involved in oxidative stress.

Systemic infection

Several chronic systemic infections have been linked with increased oxidative stress throughout the body. Human immunodeficiency virus (HIV) infection is associated with an increase in leukocyte number and activation within semen (Umapathy *et al*., 2001). Hepatitis B and C infection has also been correlated with significant hepatic oxidative stress (Chen and Siddiqui, 2007; Seronello *et al*., 2007). At present it is unknown if this oxidative stress extends to the semen, but impaired sperm motility seen in hepatitis B and C patients (Durazzo *et al*., 2006; Vicari *et al*., 2006), makes this possible. Finally, chronic infections such as tuberculosis (Srinivasan *et al*., 2004), leprosy (Vijayaraghavan *et al*., 2005), malaria (Guha *et al*., 2006) and Chagas disease (Macao *et al*., 2007) have all been linked with elevated degrees of systemic oxidative stress. While no study has directly linked these chronic infectious diseases with sperm oxidative stress, it is

unlikely that the male reproductive tract would be spared from this systemic oxidative insult.

Autoimmune/inflammatory

Chronic non-bacterial prostatitis (NIH Category III) is a chronic inflammation of the prostate in the absence of infection and has been reported by several groups to be associated with considerably elevated oxidative stress within semen (Pasqualotto *et al*., 2000; Shahed and Shoskes, 2000; Potts and Pasqualitis, 2003). Chronic non-bacterial prostatitis accounts for in excess of 90% of all cases and effects 10% of men (Schaeffer, 2003). In the majority of cases of chronic non-bacterial prostatitis it is reported that an adverse autoimmune response to seminal or prostate antigens is responsible for the pathology, leading to an increase in pro-inflammatory cytokines and activated ROS producing leukocytes within the semen (Batstone *et al*., 2002; Motrich *et al*., 2005; Motrich *et al*., 2007). While the exact trigger for this response is unknown, one report has linked a polymorphism of the TH-2 cytokine IL-10 with chronic non-bacteria prostatitis (Shoskes *et al*., 2002). A lack of this Th-2 cytokine may tip the immune balance towards the Th-1 direction leading to the generation of T lymphocytes reactive against prostate antigens. These T cells will liberate cytokines such as IFN-γ, TNF-α and IL-1β that stimulate chemotaxis and activation of leukocytes, leading to increased seminal oxidative stress (Motrich *et al*., 2005). It is therefore not surprising to see the majority of studies linking chronic non-bacterial prostatitis with a significant reduction in sperm density, motility, morphology and membrane integrity (Christiansen *et al*., 1991; Leib *et al*., 1994; Krieger *et al*., 1996; Engeler *et al*., 2003; Motrich *et al*., 2005; Henkel *et al*., 2006); although this is refuted by some groups (Pasqualotto *et al*., 2000; Ludwig *et al*., 2003).

Oxidative stress has been proposed as a significant cause for infertility after vasectomy reversal. It is believed that vasectomy disrupts the normal blood-testis barrier, leading to a loss of immune privilege and activation of immune responses against sperm (Filippini *et al*., 2001). Several studies have documented an increase in seminal leukocytes, pro-inflammatory cytokines and free radical production within semen following vasectomy reversal (Shapiro *et al*., 1998; Kolettis *et al*., 1999; Sharma *et al*., 1999; Nandipati *et al*., 2005).

Testicular

Oxidative stress is now widely believed to be the principal underlying pathology linking varicocele with male infertility (Hendin *et al*., 1999; Barbieri *et al*., 1999; Saleh *et al*., 2003b; Nallella *et al*., 2004; Smith *et al*., 2006; Agarwal *et al*., 2006c; Ishikawa *et al*., 2007; Smith *et al*., 2007). The increase in varicocele-related ROS production is strongly correlated with a reduction in sperm DNA integrity when assessed by either TUNEL (Smith *et al*., 2006) or 8-hydroxy-2′-deoxyguanosine DNA oxidative metabolite levels (Chen *et al*., 2004).

Cryptorchidism is a common cause for male factor infertility in which the primary pathology is hypo-spermatogenesis due to deficient maturation of gonocytes to type A spermatogonia (Huff *et al*., 1991). However, recently it has been reported that men with cryptorchidism surgically treated with orchidoplexy early in life still have markedly elevated sperm ROS production and DNA fragmentation compared with fertile controls (Smith *et al*., 2007).

Torsion of the spermatic cord has long been recognized as a cause of male infertility, even when this torsion is unilateral. It is now generally accepted that oxidative stress related to ischemia-reperfusion injury is the underlying cause of damage to both the torted and contra-lateral testis. A prolonged period of ischemia followed by surgical or spontaneous restoration of blood flow leads to an influx of activated leukocytes into both testis (Turner *et al*., 2004) and a consequent increase in generation of free radicals (Filho *et al*., 2004). Oxidative stress then leads to necrosis of the germinal cells with resulting subfertility or infertility.

Chronic disease

Diabetes has long been recognized to impair male fertility by interfering with both spermatogenesis and erectile function. Recently it has been reported that diabetic men have significantly higher levels of sperm DNA fragmentation than normal controls (Agbaje *et al*., 2007). While this study did not directly measure oxidative stress, the authors proposed that the most likely mechanism for the observed increase in sperm DNA damage was an increase in oxidative stress as this is now recognized as a key pathology underlying many chronic complications of diabetes. In support, studies using the Streptozotocin-induced diabetic rat model have found a significant increase in testicular oxidative stress within 6 weeks of initiation of the diabetic state (Shrilatha and Muralidhara, 2007).

Chronic inflammation and oxidative stress are highly prevalent in patients with chronic kidney disease and end-stage renal disease (Oberg *et al*., 2004). Surprisingly, even when uraemia is reversed by haemodialysis, a persisting state of chronic inflammation and oxidative stress persists (Danielski *et al*., 2003; Pupim *et al*., 2004). Furthermore, renal transplant patients with stable renal function and no obvious signs of immune rejection of their graft also have elevated levels of oxidative stress (Moreno *et al*., 2005).

Patients with haemaglobinopathies such as beta-thalassemia major have high degrees of systemic oxidative stress (Livrea *et al*., 1996), with this oxidative damage confirmed to involve sperm (Carpino *et al*., 2004). The likely cause of oxidative stress is iron overload from multiple blood transfusions. Iron is a potent pro-oxidant capable of redox cycling when not safely bound to transferrin in the blood or stored as ferritin in tissue.

The toxic accumulation of homocysteine may cause reproductive dysfunction and oxidative stress within the testis (Forges *et al*., 2007; Sonmez *et al*., 2007). Hyper-homocysteinaemia usually occurs due to suboptimal re-methylation of homocysteine to methionine by the enzyme methyl tetrahydrofolate reductatse (MTHFR) caused by a dietary deficiency of folate or a single-nucleotide polymorphism (SNP) in the MTHFR gene (Selhub, 1999; Matthews, 2002). Several investigators have reported that SNPs (C677T and others) in the MTHFR gene are more commonly found in the infertile men (Bezold *et al*., 2001; Park *et al*., 2005; Lee *et al*., 2006; Zhou-Cun *et al*., 2007), placing these men at increased risk of homocysteine-induced oxidative stress.

Laboratory identification of oxidative stress-related male infertility

One of the main reasons why screening for oxidative stress is not routine in andrology laboratories is the cost and complexity of testing and the lack of a single standardized measure of oxidative

stress. At present there are over 30 assays of oxidative stress (Ochsendorf, 1999), broadly divided into three different types. This review will focus on the most popular and clinically useful assays currently being performed.

Direct methods

These assays measure damage created by excess free radicals against the sperm lipid membrane or DNA. As oxidative stress is the result of an in balance between ROS production and total antioxidant capacity (TAC), direct tests reflect the net biological effect between these two opposing forces.

The most widely used method of assessing sperm membrane peroxidation is the measurement of MDA levels in sperm or seminal plasma with the thiobarbituric acid assay. MDA levels in sperm are quite low and therefore require the use of sensitive high-pressure liquid chromatography (HPLC) equipment (Li *et al*., 2004; Shang *et al*., 2004) or the use of iron-based promoters and spectrofluometry measurement (Aitken *et al*., 1993). Seminal plasma levels of MDA are 5–10-fold higher than sperm, making measurement on standard spectrophotometers possible (Sanocka *et al*., 1997; Nakamura *et al*., 2002; Tavilani *et al*., 2005). Measurement of MDA appears to be of some clinical relevance since its concentration within both seminal plasma and sperm is elevated in infertile men with excess ROS production, compared with fertile controls or normozoospermic individuals (Sanocka *et al*., 1997; Nakamura *et al*., 2002; Tavilani *et al*., 2005; Hsieh *et al*., 2006). Furthermore, *in vitro* impairment of motility, sperm DNA integrity and sperm–oocyte fusion capacity by ROS is accompanied by an increase in MDA concentration (Aitken *et al*., 1989, 1993). Other direct tests of sperm membrane lipid peroxidation such as measurement of the isoprostane 8-Iso-PGF2α (Khosrowbeygi and Zarghami, 2007) and the c11-BODIPY assay (Aitken *et al*., 2007; Kao *et al*., 2007) are showing promise but are not yet in common usage.

It is well recognized that oxidative stress is one of the major causes of sperm DNA damage (Aitken *et al*., 1998; Oger *et al*., 2003; Saleh *et al*., 2003a, b). However, measurement of sperm DNA damage by TUNEL or SCSA is an imperfect assessment of oxidative stress as sperm DNA can be damaged by non-oxidative mechanisms such as aberrant apoptosis and incomplete sperm protamination (Ozmen *et al*., 2007). The best direct assessment of sperm DNA oxidative damage is the measurement of the oxidized deoxynucleoside, 8-oxo-7,8-dihydro 2′ deoxyguanosine (8-OHdG). This can be measured in sperm or seminal plasma by HPLC (Fraga *et al*., 1991; Loft *et al*., 2003), enzyme-linked immunoabsorbent assay (Nakamura *et al*., 2002) or directly within sperm using immunoflurorescence (Kao *et al*., 2007). Since a large prospective study has reported that chances of natural conception is inversely correlated with sperm 8-OHdG levels (Loft *et al*., 2003), measurement of this direct marker of sperm oxidative stress appears to have some clinical utility.

Indirect methods

Chemoluminescence assays using either Luminol or Lucigenin are the most commonly described technique to detect ROS production within semen. These probes are very sensitive and have the advantage of relatively well established reported ranges for both the fertile and infertile population (Ochsendorf *et al*., 1994; Williams and Ford, 2005; Athayde *et al*., 2007). However, general uptake by clinical andrology laboratories has been hampered by expensive equipment (luminometer) and difficulties with quality control created by assay confounders such as incubation time, leukocyte contamination and presence of seminal plasma contamination (Kobayashi *et al*., 2001; Aitken *et al*., 2004). Furthermore, Lucigenin has been shown to undergo auto-oxidization which itself leads to the production of superoxide anions (Liochev and Fridovich, 1997). This makes chemoluminescent probes such as Lucigenin less than ideal reagents for measurement of sperm superoxide anion production. A simpler alternative may be light microscopy quantification of nitroblue tetrazolium (NBT) activity. NBT is a yellow water soluble compound that reacts with superoxide anions within cells to produce a blue pigment diformazan. The amount of diformazan crystals seen within a leukocyte or sperm reflects its superoxide anion production. The NBT assay has been shown to correlate well with traditional chemoluminescence techniques (Esfandiari *et al*., 2003) but has two distinct advantages. First, the NBT assay is inexpensive to set up as it only requires a light microscope. Secondly, the NBT assay can discriminate between production of ROS by sperm and leukocytes without the need for addition of activating peptides (FMLP) used in chemoluminescence assays (WHO manual, 1999).

Measurement of TAC within semen can be conducted in a variety of ways. The ability of seminal plasma to inhibit chemoluminescence elicited by a constant source of ROS (horse-radish peroxidase) is a commonly used technique. The TAC is usually quantified against a Vitamin E analogue (Trolox) and expressed as a ROS-TAC score (Sharma *et al*., 1999). However, colourimetry techniques based on the colour change of ABTS (2,2′-azinobis3-ethylbenzo-thiazoline-6-sulphate) are now becoming more popular as they are cheaper and easier to perform (Said *et al*., 2003; Erel, 2004). The reduced ABTS molecule is oxidized to ABTS+ using hydrogen peroxide and a peroxidase to form a relatively stable blue-green colour measured at 600 nm with a standard spectrophotometer. Antioxidants present within seminal plasma suppress this colour change to a degree that is proportional to their concentrations. Again the antioxidant activity is quantified using Trolox.

Oxidative stress implied from routine semen analysis

A summary of the routine laboratory test ‘sentinel signs’ suggesting the possible presence of sperm oxidative stress is contained in Table I. While a reduction in any of the sperm parameters (count, motility, morphology) is more commonly seen in men with oxidative stress, asthenozoospermia is probably the best surrogate marker for oxidative stress in a routine semen analysis (Aitken and Baker, 1995; Aitken *et al*., 1995a, b; Whittington *et al*., 1999; Keskes-Ammar *et al*., 2003; Kao *et al*., 2007). A link between impaired sperm motility and oxidative stress also extends to the sperm DNA as a recent study has identified a highly significant correlation between oxidation of sperm DNA and reduced motility (Kao *et al*., 2007).

Hyperviscosity of seminal plasma is associated with increased levels of seminal plasma MDA (Aydemir *et al*., 2008) and reduced seminal plasma antioxidant status (Siciliano *et al*., 2001), making impaired viscosity a reasonable surrogate marker of oxidative stress. Infection of the semen with *Ureaplasma urealyticum* is associated with increased seminal plasma viscosity

Table I. Sentinel laboratory signs suggesting possible sperm oxidative stress.

1. Poor sperm motility.
2. Teratozoospermia.
3. High number of round cells (? Leukocytes) in semen.
4. Increased semen viscosity.
5. Poor sperm membrane integrity on hypo-osmolar swelling test (HOST).
6. Poor fertilization on routine IVF.
7. Poor sperm motility after overnight incubation with the oocyte.
8. Poor blastocyst development in the absence of a clear female factor (advanced maternal age/poor ovarian reserve).

(Wang *et al*., 2006) and an increase in ROS production (Potts *et al*., 2000a, b). It is possible that these infections may damage the prostate and seminal vesicle, altering the substrates required for creation of normal semen viscosity.

A large number of round cells within semen may suggest the presence of oxidative stress as they may represent seminal leukocytes (Sharma *et al*., 2001). However, round cells may also be immature sperm rather than leukocytes, so formal identification of leukocytes requires ancillary tests such as the peroxidase test, CD45 staining or measurement of seminal elastase (WHO manual, 1999; Zorn *et al*., 2003b; Kopa *et al*., 2005). Finally, poor sperm membrane integrity assessed by the hypo-osmolar swelling test has been linked with the presence of sperm oxidative stress (Dandekar *et al*., 2002).

Management of oxidative stress related infertility

Once an individual has been identified as having oxidative stress related infertility, treatment should be aimed at identification and amelioration of the underlying cause before considering antioxidant treatment. The following paragraphs are the author's suggestions for investigation and management based on the underlying causes of oxidative stress outlined in previous paragraphs. These recommendations are summarized in Table II.

Lifestyle modification

Lifestyle behaviours such as smoking, poor diet, alcohol abuse, obesity or psychological stress have all been linked with oxidative stress. While the effectiveness of elimination of these lifestyle triggers for oxidative stress has not been formally tested, it is likely that making positive lifestyle changes such as a diet high in fruit/vegetables, maintenance of normal weight and a reduction in smoking/alcohol intake would have at least some beneficial effect on sperm health.

Environmental exposures

Exposure to heat, pollution and toxins (heavy metals and plasticizers) have all been linked with oxidative stress. Men should be advised to avoid activities which may heat the scrotum such as long baths and saunas. Proper ventilation and use of personal protective equipment at work will hopefully reduce men's exposure to chemical and metal vapours linked with oxidative stress.

Treatment of infection/inflammation

Infection of the semen and male accessory sex glands with *Chlamydia* and *Ureaplasma* has been conclusively linked with an increase in oxidative stress. As both of these infections are treatable with antibiotics, it makes sense to screen all men with known oxidative stress for these bacterial pathogens. Two studies have now confirmed the ability of antibiotic treatment to reduce sperm oxidative stress and subsequently improve sperm quality (Omu *et al*., 1998; Vicari, 2000). One relatively large and well-conducted study randomized men with *Chlamydia* or *Ureaplasma* infection to either 3 months of antibiotics or no treatment (Vicari, 2000). Compared with the controls, the antibiotic treated group exhibited a significant fall in seminal leukocytes and ROS production at 3 months, an improvement in sperm motility and a significant improvement in natural conception (28.2 versus 5.4%, $P = 0.009$). A smaller study using only 10 days of antibiotic treatment did not produce any significant decline in seminal leukocyte count or improvement in motility (Krause *et al*., 2003). While this study did not measure ROS production in semen, it is likely that prolonged courses of antibiotics (3 months) are required to completely irradiate difficult-to-treat male accessory gland infections and reverse oxidative pathology.

In addition to antibiotic treatment, non-steroidal anti-inflammatory (NSAID) drugs may also reduce seminal leukocytes production of free radicals. In one study men with antibiotic treated *Chlamydia* or *Ureaplasma* infection were randomized to either a NSAID or carnitine antioxidant and monitored for improvements in sperm quality over the next 4 months (Vicari *et al*., 2002). Those men treated with 2 months of NSAID followed by 2 months of carnitine had the most significant reduction in seminal ROS production and improvement in sperm motility/viability. In addition, a one month course of a COX-2 anti-inflammatory has been shown to significantly reduce sperm leukocyte count, while improving sperm motility, morphology and viability (Gambera *et al*., 2007). It would therefore appear that a combination of antibiotics followed by a course of anti-inflammatory medication is the preferred treatment path in infection related oxidative stress.

Table II. Summary of treatment options in male oxidative infertility.

1. Minimize 'lifestyle' triggers of oxidative stress. This may include stopping smoking, improved diet, losing weight.
2. Minimize environmental exposure to heat, pollutions and toxins.
3. Direct treatment of the underlying stimulus for sperm oxidative stress. For example, antibiotic treatment of *Chlamydia* or *Mycoplasma* infection.
4. Surgery. This would include ligation of a varicocele or the use of testicular derived sperm during IVF to improve sperm DNA quality.
5. Vitamin and antioxidant supplements, with or without the addition of anti-inflammatory medications to decrease leukocyte ROS production.
6. Surgical extraction of sperm. If conservative methods such as lifestyle modification, antioxidant therapy fail use of testicular sperm extraction may be justified.
7. Optimize laboratory procedures. Minimization of iatrogenic oxidative stress can be achieved by limiting semen centrifugation times and avoidance of use of cryo-preserved sperm if possible.

Direct treatment of oxidative pathology

Several investigators have reported that surgical treatment of a varicocoele can reduce seminal ROS levels and improve sperm DNA integrity (Mostafa *et al*., 2001; Zini *et al*., 2005; Hurtado de Catalfo *et al*., 2007; Werthman *et al*., 2007). While the most

recent meta-analysis examining the effect of varicocelectomy on spontaneous conception shows a significant benefit (Marmar *et al*., 2007), the Cochrane Database suggests that there is no benefit (Evers and Collins, 2004). Well-conducted randomized studies measuring oxidative end-points (sperm lipid peroxidation and oxidative DNA damage) and pregnancy rates need to be performed before routine use of varicocelectomy can be advocated in men with oxidative stress. Until these studies become available, selective ligation of grade II/III varicoceles in men with poor reproductive outcomes despite oral antioxidant therapy is probably reasonable practice.

Vitamin and antioxidant supplementation

Elevated homocysteine has been linked with oxidative stress. The B group vitamins folate, Vitamin B_6 and Vitamin B_{12} are known to increase the enzymatic efficiency of the MTHFR and cystathionine β-synthase enzymes responsible for removing homocysteine from the circulation (Matthews, 2002). While yet to be proven to enhance sperm quality, the use of a B group vitamin supplement (5 mg folate, 100 mg Vitamin B_6 and 100 μg Vitamin B_{12}) is probably warranted in any man found to have hyper-homocysteinaemia and oxidative stress as this treatment is inexpensive and without significant side effects.

To date, over 30 studies have been published examining the effect of various antioxidant treatments on sperm parameters and pregnancy outcome. With such a large body of evidence it would be expected that firm conclusions regarding the clinical effectiveness of oral antioxidants on sperm function and pregnancy outcome would be available. Unfortunately this is not the case because of the use of different types and doses of antioxidants, lack of proper prospective placebo controlled study design and small sample sizes. Many small non-controlled trials report significant improvements in sperm count, motility and morphology while on antioxidant therapy (reviewed in Agarwal *et al*., 2004). However, as these studies are open to bias this review will only consider properly conducted placebo controlled trials or prospective trials measuring oxidative stress end points (sperm peroxidation and DNA damage).

Several studies have reported that levels of ROS within semen can be reduced by augmenting the scavenging capacity of seminal plasma using oral antioxidant supplements. The oral antioxidant Astaxanthin (Comhaire *et al*., 2005), carnitine (Vicari and Calogero, 2001) or a combination of antioxidants such as acetylcysteine, β-carotene, Vitamin E and essential fatty acids (Comhaire *et al*., 2000) have all been shown to directly reduce seminal ROS levels. A randomized control study comparing 3 months of Vitamin E (600 mg/day) treatment with placebo has confirmed this reduction in seminal ROS levels (Kessopoulou *et al*., 1995). Furthermore, a combination of 400 mg of Vitamin E and 225 μg of selenium (Keskes-Ammar *et al*., 2003) or 300 mg of Vitamin E alone (Suleiman *et al*., 1996) have been shown in placebo controlled studies to reduce sperm MDA levels. Finally, a well-designed RCT of 2 months treatment with 1 g of Vitamin C and Vitamin E reported a very significant reduction in sperm DNA damage (Greco *et al*., 2005a, b). This finding is supported by non-controlled studies which have also reported a reduction in sperm DNA damage with the use of a combination of Vitamin C and E (400 mg each), β-carotene (18 mg), zinc and selenium (Menezo *et al*., 2007) or a combination of acetylcysteine, 180 mg Vitamin E, 30 mg β-carotene and essential fatty acids (Comhaire *et al*., 2000).

While many relatively poorly designed studies have shown antioxidant supplements to boost sperm count and morphology, the majority of good-quality studies do not (Agarwal *et al*., 2004). The only parameter that appears to be possibly improved with oral antioxidant therapy is sperm motility. Many well-conducted studies have shown small but significant improvements in sperm motility with supplementation of carnitine (Lenzi *et al*., 2004; Balercia *et al*., 2005), selenium (Scott *et al*., 1998), Vitamin E (Suleiman *et al*., 1996), Vitamin E and selenium (Keskes-Ammar *et al*., 2003), glutathione (Lenzi *et al*., 1993) and Astaxanthin (Comhaire *et al*., 2005). However, two prospective RCT comparing Vitamin C and E supplementation with placebo have found antioxidants to have no ability to improve sperm motility (Rolf *et al*., 1999; Greco *et al*., 2005a).

While many studies have show improvements in sperm quality with antioxidant treatment, the ability of these changes to translate into improved chances of pregnancy is less clear. Suleiman *et al*. (1996) reported that treatment with Vitamin E resulted in a significant fall in ROS damage to sperm and an improvement in spontaneous pregnancy rates during the next 6 months (21% pregnant rate in the Vitamin E group V 0% placebo). Conversely, Rolf *et al*. (1999) did not report any improvement in spontaneous pregnancy outcome from 2 months treatment with a combination of Vitamin C and Vitamin E. Finally, a recent RCT comparing the antioxidant formulation Menevit with placebo reported a significant increase in clinical pregnancy rate if the antioxidant was taken for 3 months prior to IVF-ICSI treatment (Tremellen *et al*., 2007). The Menevit nutraceutical is postulated to improve sperm quality by three complimentary mechanisms. First, it contains traditional antioxidants such as Vitamins C and E, selenium and lycopene to protect sperm from ROS already produced. Second, it contains garlic which is known to have an anti-inflammatory effect, thereby potentially reducing seminal leukocyte ROS production (Hodge *et al*., 2002; Chang *et al*., 2005). Finally, Menevit contains zinc, selenium and folate that are believed to play a role in augmenting protamine packaging of sperm DNA (Kvist *et al*., 1987; Pfeifer *et al*., 2001), helping to protect sperm from ROS attack. While it is yet to be proven that combinational therapy such as Menevit improves sperm DNA integrity, it appears logical that using several antioxidants with different modes of action, together with an agent to reduce leukocyte ROS production (Vicari *et al*., 2002; Gambera *et al*., 2007; Tremellen *et al*., 2007) is most likely to result in a beneficial effect.

Surgical extraction of sperm

It has been suggested that while sperm are in contact with Sertoli cells they are relatively protected from oxidative attack (Greco *et al*., 2005b), with most ROS-mediated damage occurring during storage in the epididymis (Greco *et al*., 2005b). Two studies have compared sperm DNA quality in the same individual using either ejaculate (Greco *et al*., 2005a, b) or surgically aspirated epididymal sperm (O'Connell *et al*., 2002) with sperm surgically extracted from the testicle. Both of these studies report significant improvements in sperm DNA quality in the testicle

Table III. Summary of the evidence linking OS with male infertility.

1. Many infertile men have significantly higher levels of ROS within their semen compared to fertile men, placing them at increased risk of OS.	Iwasaki and Gagnon, 1992; Zini *et al*., 1993; Ochsendorf *et al*., 1994; Shekarriz *et al*., 1995a, b; Pasqualotto *et al*., 2001; Agarwal *et al*., 2006a, b; Athayde *et al*., 2007.
2. Many infertile men have significantly lower levels of protective antioxidants within their semen compared to fertile men, placing them at increased risk OS.	Jeulin *et al*., 1989; Fraga *et al*., 1996; Smith *et al*., 1996; Therond *et al*., 1996; Alkan *et al*., 1997; Lewis *et al*., 1997; Miesel *et al*. 1997; Sanocka *et al*., 1997; Giannattasio *et al*., 2002; Koca *et al*., 2003; Garrido *et al*., 2004a, b; Mostafa *et al*., 2006; Khosrowbeygi and Zarghami, 2007.
3. The generation of sperm OS *in vitro* (direct application of ROS or stimulation of sperm intrinsic ROS production) is associated with biochemical evidence of sperm lipid peroxidation and decreased sperm motility/oocyte fertilization capacity.	Jones *et al*., 1979; Aitken *et al*., 1989; Aitken and Baker, 1995; Aitken *et al*., 1995a, b, 1998; Twigg *et al*., 1998; Whittington and Ford, 1998; Kemal Duru *et al*., 2000.
4. The addition of antioxidants to culture media protects sperm from OS mediated impaired motility.	MacLeod, 1943; Kobayashi *et al*., 1991; Oeda *et al*., 1997; Zheng and Zhang, 1997; Donnelly *et al*., 2000; Rossi *et al*. 2001; Yenilmez *et al*., 2006.
5. Seminal OS in infertile men is correlated with impaired sperm motility/fertilization capacity and increased sperm membrane oxidation.	Aitken *et al*., 1989; Saleh *et al*., 2003a, b; Zorn *et al*., 2003a, b; Zalata *et al*., 2004; Jedrzejczak *et al*., 2005; Kao *et al*., 2007; Khosrowbeygi and Zarghami, 2007.
6. Antioxidant treatment of infertile men can significantly improve sperm motility.	Lenzi *et al*., 1993, 2004; Suleiman *et al*., 1996; Scott *et al*., 1998; Keskes-Ammar *et al*., 2003; Balercia *et al*., 2005.
7. The generation of sperm OS *in vitro* (direct application of ROS or stimulation of sperm intrinsic ROS production) is associated with an increase in sperm DNA damage.	Aitken *et al*., 1998; Twigg *et al*., 1998; Kemal Duru *et al*., 2000.
8. Seminal OS in infertile men is correlated with an increase in sperm DNA damage.	Kodama *et al*., 1997; Nakamura *et al*., 2002; Saleh *et al*., 2002b; Loft *et al*., 2003; Oger *et al*., 2003; Wang *et al*., 2003; Moustafa *et al*., 2004; Henkel *et al*., 2005; Kao *et al*., 2007.
9. Antioxidant treatment of infertile men can significantly improve sperm DNA quality.	Kodama *et al*., 1997; Comhaire *et al*. 2000; Greco *et al*., 2005a, b; Menezo *et al*., 2007.
10. The use of antioxidant supplements by infertile men can significantly increase their partners chances of spontaneous or IVF assisted pregnancy (RCTs only).	Suleiman *et al*., 1996; Tremellen *et al*., 2007.

OS, oxidative stress.

derived samples. Unfortunately neither of these studies assessed oxidative damage to sperm so it is presently uncertain if protection from epididymal oxidative stress is the sole reason for the observed improvements in DNA quality. As such, resort to the use of testicular derived sperm in men with poor DNA quality should only occur if more conservative treatments such as lifestyle modification and antioxidant therapy have failed.

Laboratory techniques to reduce the effects of oxidative stress

Centrifugation of a semen sample prior to its use in IUI or IVF can exacerbate sperm oxidative stress. This can be limited by reducing the time that the semen is centrifuged (Shekarriz *et al*., 1995a, b), use of non-centrifuge separation techniques such as 'swim-up' or glass-wool filtration and limiting the time in which sperm are cultured in media away from seminal plasma. Furthermore, culturing sperm under low oxygen tension (5%O_2/95% CO_2 versus 20% atmospheric O_2 content) has been shown to significantly improve sperm quality by reducing seminal leukocyte ROS production (Griveau and Le Lannou, 1997; Whittington and Ford, 1998). Avoiding use of cryopreserved sperm for fertilization is also ideal since ROS are produced during freezing and thawing of the sperm, thereby decreasing sperm quality (Watson, 2000).

Sperm preparation media may also be supplemented with a variety of antioxidants to guard against oxidative stress. The addition of catalase/SOD (Rossi *et al*., 2001), Vitamin C (Donnelly *et al*., 1999), Vitamin E (Donnelly *et al*., 1999; Yenilmez *et al*., 2006), ferulic acid (Zheng and Zhang, 1997), EDTA (Gomez and Aitken, 1996; Gomez *et al*., 1996), glutathione/hypotaurine (Donnelly *et al*., 2000), albumin (Twigg *et al*., 1998) and *N*-acetyl-cysteine (Oeda *et al*., 1997) to sperm preparation media have all been shown to protect sperm from oxidative attack. At the present moment commercial sperm preparation media does not contain any antioxidants aside from albumin and amino acids. Optimized culture media for sperm is unfortunately lagging well behind the complex sequential media developed for embryos and certainly needs intensive research as soon as possible.

Overview

An expanding body of evidence now supports a role for oxidative stress as a significant cause of male infertility (summarized in Table III). However, despite being a common pathology in infertile men, oxidative stress is ignored by many infertility practitioners. The currently popular response of resorting to mechanical techniques such as IVF-ICSI in all cases of male factor infertility is unlikely to be 'best practice' since ROS damaged paternal DNA will result in poor quality blastocysts, less than optimal pregnancy rates and an increase in miscarriage. Antioxidant supplements have now been shown in randomized placebo controlled studies to protect sperm from oxidative related DNA damage and to boost pregnancy rates. It may therefore be prudent to consider using antioxidants in all infertile men exhibiting oxidative stress. Presently, one-third of men in infertile relationships already take such therapies (Zini *et al*., 2004), indicating patient acceptance of antioxidant supplementation in combination with traditional ART treatments. Of

course, antioxidants should be offered in combination with changes in lifestyle such as avoiding toxins (cigarette smoke, pollutants, heavy metals) and excessive heat.

While a role for oxidative stress in male infertility is now established, many unanswered questions still remain. First, there is a clear need to develop inexpensive assays to identify sperm oxidative stress that can be easily conducted in any andrology laboratory. Secondly, large RCTs are needed to confirm the effectiveness of surgical interventions (varicocelectomy, testicular biopsy) in the management of oxidative stress. Further research is also required to determine what combination and dose of antioxidant supplement provides sperm with maximal protection against oxidative stress. Finally, the development of new sperm culture media that can better protect sperm from the ravages of ROS damage is clearly required.

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