

# 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<sub>2</sub>) forms the superoxide anion radical (O<sub>2</sub><sup>•-</sup>), 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 (•OH), peroxy radical (ROO•) or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). 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<sub>2</sub>O<sub>2</sub>) 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, peroxy-nitrite, nitroxyl anion and peroxy-nitrous 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).

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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 TNF $\alpha$  (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  $\beta$ -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),  $\alpha$ -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 (prostatasomes) 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).

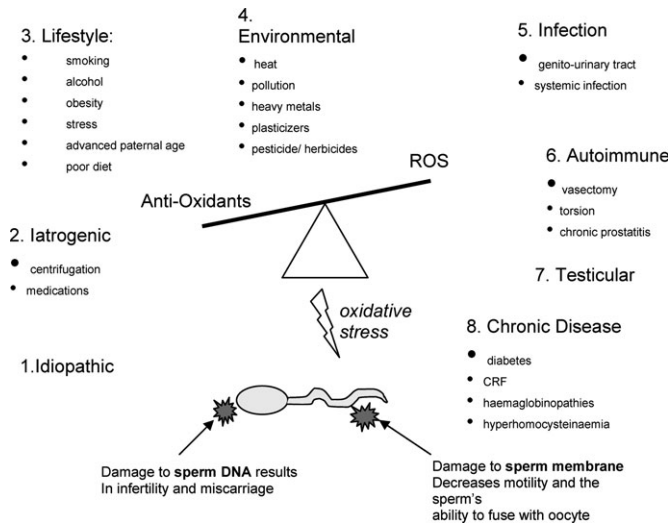


Figure 1: The oxidative stress balance.

## 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; Shekarri *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,  $\beta$ -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 (Eskioçak *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 phthalate 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. pyogenes*), 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 affects 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- $\gamma$ , TNF- $\alpha$  and IL-1 $\beta$  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 orchidopexy 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 haemoglobinopathies 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 reductase (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 imbalance 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-PGF $2\alpha$  (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 immunosorbent assay (Nakamura *et al.*, 2002) or directly within sperm using immunofluorescence (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-oxidation 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'-azino-bis(3-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

**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.

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.

#### Direct treatment of oxidative pathology

Several investigators have reported that surgical treatment of a varicocele 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<sub>6</sub> and Vitamin B<sub>12</sub> are known to increase the enzymatic efficiency of the MTHFR and cystathionine  $\beta$ -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<sub>6</sub> and 100  $\mu$ g Vitamin B<sub>12</sub>) 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,  $\beta$ -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  $\mu$ g 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),  $\beta$ -carotene (18 mg), zinc and

selenium (Menezes *et al.*, 2007) or a combination of acetylcysteine, 180 mg Vitamin E, 30 mg  $\beta$ -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 <i>et al.</i> , 1993; Ochsendorf <i>et al.</i> , 1994; Shekarriz <i>et al.</i> , 1995a, b; Pasqualotto <i>et al.</i> , 2001; Agarwal <i>et al.</i> , 2006a, b; Athayde <i>et al.</i> , 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 <i>et al.</i> , 1989; Fraga <i>et al.</i> , 1996; Smith <i>et al.</i> , 1996; Therond <i>et al.</i> , 1996; Alkan <i>et al.</i> , 1997; Lewis <i>et al.</i> , 1997; Miesel <i>et al.</i> , 1997; Sanocka <i>et al.</i> , 1997; Giannattasio <i>et al.</i> , 2002; Koca <i>et al.</i> , 2003; Garrido <i>et al.</i> , 2004a, b; Mostafa <i>et al.</i> , 2006; Khosrowbeygi and Zarghami, 2007.
3. The generation of sperm OS <i>in vitro</i> (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 <i>et al.</i> , 1979; Aitken <i>et al.</i> , 1989; Aitken and Baker, 1995; Aitken <i>et al.</i> , 1995a, b, 1998; Twigg <i>et al.</i> , 1998; Whittington and Ford, 1998; Kemal Duru <i>et al.</i> , 2000.
4. The addition of antioxidants to culture media protects sperm from OS mediated impaired motility.	MacLeod, 1943; Kobayashi <i>et al.</i> , 1991; Oeda <i>et al.</i> , 1997; Zheng and Zhang, 1997; Donnelly <i>et al.</i> , 2000; Rossi <i>et al.</i> , 2001; Yenilmez <i>et al.</i> , 2006.
5. Seminal OS in infertile men is correlated with impaired sperm motility/fertilization capacity and increased sperm membrane oxidation.	Aitken <i>et al.</i> , 1989; Saleh <i>et al.</i> , 2003a, b; Zorn <i>et al.</i> , 2003a, b; Zalata <i>et al.</i> , 2004; Jedrzejczak <i>et al.</i> , 2005; Kao <i>et al.</i> , 2007; Khosrowbeygi and Zarghami, 2007.
6. Antioxidant treatment of infertile men can significantly improve sperm motility.	Lenzi <i>et al.</i> , 1993, 2004; Suleiman <i>et al.</i> , 1996; Scott <i>et al.</i> , 1998; Keskes-Ammar <i>et al.</i> , 2003; Balercia <i>et al.</i> , 2005.
7. The generation of sperm OS <i>in vitro</i> (direct application of ROS or stimulation of sperm intrinsic ROS production) is associated with an increase in sperm DNA damage.	Aitken <i>et al.</i> , 1998; Twigg <i>et al.</i> , 1998; Kemal Duru <i>et al.</i> , 2000.
8. Seminal OS in infertile men is correlated with an increase in sperm DNA damage.	Kodama <i>et al.</i> , 1997; Nakamura <i>et al.</i> , 2002; Saleh <i>et al.</i> , 2002b; Loft <i>et al.</i> , 2003; Oger <i>et al.</i> , 2003; Wang <i>et al.</i> , 2003; Moustafa <i>et al.</i> , 2004; Henkel <i>et al.</i> , 2005; Kao <i>et al.</i> , 2007.
9. Antioxidant treatment of infertile men can significantly improve sperm DNA quality.	Kodama <i>et al.</i> , 1997; Comhaire <i>et al.</i> , 2000; Greco <i>et al.</i> , 2005a, b; Menezo <i>et al.</i> , 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 <i>et al.</i> , 1996; Tremellen <i>et al.</i> , 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<sub>2</sub>/95% CO<sub>2</sub> versus 20% atmospheric O<sub>2</sub> 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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## References

- Acharya UR, Acharya S, Mishra M. Lead acetate induced cytotoxicity in male germinal cells of Swiss mice. *Ind Health* 2003;**41**:291–294.
- Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. *BJU Int* 2005;**95**:503–507.
- Agarwal DK, Maronpot RR, Lamb JCT, Kluwe WM. Adverse effects of butyl benzyl phthalate on the reproductive and hematopoietic systems of male rats. *Toxicology* 1985;**35**:189–206.
- Agarwal A, Nallella KP, Allamaneni SS, Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomed Online* 2004;**8**:616–627.
- Agarwal A, Prabakaran S, Allamaneni S. What an andrologist/urologist should know about free radicals and why. *Urology* 2006a;**67**:2–8.
- Agarwal A, Sharma RK, Nallella KP, Thomas AJ, Jr, Alvarez JG, Sikka SC. Reactive oxygen species as an independent marker of male factor infertility. *Fertil Steril* 2006b;**86**:878–885.
- Agarwal A, Prabakaran S, Allamaneni SS. Relationship between oxidative stress, varicocele and infertility: a meta-analysis. *Reprod Biomed Online* 2006c;**12**:630–633.
- Agbaje IM, Rogers DA, McVicar CM, McClure N, Atkinson AB, Mallidis C, Lewis SE. Insulin dependant diabetes mellitus: implications for male reproductive function. *Hum Reprod* 2007;**22**:1871–1877.
- Aitken RJ, Baker HW. Seminal leukocytes: passengers, terrorists or good samaritans? *Hum Reprod* 1995a;**10**:1736–1739.
- Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001;**122**:497–506.
- Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 1989;**41**:183–197.
- Aitken RJ, Harkiss D, Buckingham D. Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J Reprod Fertil* 1993;**98**:257–265.
- Aitken RJ, West K, Buckingham D. Leukocytic infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. *J Androl* 1994;**15**:343–352.
- Aitken RJ, Buckingham DW, Brindle J, Gomez E, Baker HW, Irvine DS. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Hum Reprod* 1995b;**10**:2061–2071.
- Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci* 1995c;**108**:2017–2025.
- Aitken RJ, Buckingham DW, West K, Brindle J. On the use of paramagnetic beads and ferrofluids to assess and eliminate the leukocytic contribution to oxygen radical generation by human sperm suspensions. *Am J Reprod Immunol* 1996;**35**:541–551.
- Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998;**59**:1037–1046.
- Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* 2003;**7**:65–70.
- Aitken RJ, Baker MA, O'Bryan M. Shedding light on chemiluminescence: the application of chemiluminescence in diagnostic andrology. *J Androl* 2004;**25**:455–465.
- Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. *Mol Hum Reprod* 2007;**13**:203–211.
- Alaghmand M, Blough NV. Source-dependent variation in hydroxyl radical production by airborne particulate matter. *Environ Sci Technol* 2007;**41**:2364–2370.
- Alkan I, Simsek F, Haklar G, Kervancioglu E, Ozveri H, Yalcin S, Akdas A. Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. *J Urol* 1997;**157**:140–143.
- Armstrong JS, Bivalacqua TJ, Chamulitrat W, Sikka S, Hellstrom WJG. A comparison of the NADPH oxidase in human sperm and white blood cells. *Int J Androl* 2002;**25**:223–229.
- Athayde KS, Cocuzza M, Agarwal A, Krajcir N, Lucon AM, Srougi M, Hallak J. Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. *J Androl* 2007;**28**:613–620.
- Aydemir B, Onaran I, Kiziler AR, Alici B, Akyolcu MC. The influence of oxidative damage on viscosity of seminal fluid in infertile men. *J Androl* 2008;**29**:41–46.
- Baker MA, Krutskikh A, Aitken RJ. Biochemical entities involved in reactive oxygen species generation by human spermatozoa. *Protoplasma* 2003;**221**:145–151.
- Balercia G, Regoli F, Armeni T, Koverech A, Mantero F, Boscaro M. Placebo-controlled double-blind randomized trial on the use of L-carnitine, L-acetylcarnitine, or combined L-carnitine and L-acetylcarnitine in men with idiopathic asthenozoospermia. *Fertil Steril* 2005;**84**:662–671.
- Banfi B, Molnar G, Maturana A, Steger K, Demareux N, Krause KH. A Ca (2+) activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem* 2001;**276**:37594–37601.
- Banks S, King SA, Irvine DS, Saunders PT. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. *Reproduction* 2005;**129**:505–514.
- Barbieri ER, Hidalgo ME, Venegas A, Smith R, Lissi EA. Varicocele-associated decrease in antioxidant defenses. *J Androl* 1999;**20**:713–717.
- Batstone GR, Doble A, Gaston JS. Autoimmune T cell responses to seminal plasma in chronic pelvic pain syndrome (CPPS). *Clin Exp Immunol* 2002;**128**:302–307.
- Bezold G, Lange M, Peter RU. Homozygous methylenetetrahydrofolate reductase C677T mutation and male infertility. *N Engl J Med* 2001;**344**:1172–1173.
- Bezold G, Politch JA, Kiviat NB, Kuypers JM, Wolff H, Anderson DJ. Prevalence of sexually transmissible pathogens in semen from asymptomatic male infertility patients with and without leukocytospermia. *Fertil Steril* 2007;**87**:1087–1097.
- Bonde JP. The risk of male subfecundity attributable to welding of metals. Studies of semen quality, infertility, fertility, adverse pregnancy outcome and childhood malignancy. *Int J Androl* 1993;**16**(Suppl 1):1–29.
- Brackett NL, Ibrahim E, Grotas JA, Aballa TC, Lynne CM. Higher sperm DNA damage in semen from men with spinal cord injuries compared to controls. *J Androl* 2008;**29**:93–99.
- Camejo MI, Segnini A, Proverbio F. Interleukin-6 (IL-6) in seminal plasma of infertile men, and lipid peroxidation of their sperm. *Arch Androl* 2001;**47**:97–101.
- Carpino A, Tarantino P, Rago V, De Sanctis V, Siciliano L. Antioxidant capacity in seminal plasma of transfusion-dependent beta-thalassemic patients. *Exp Clin Endocrinol Diabetes* 2004;**112**:131–134.
- Chang HP, Huang SY, Chen YH. Modulation of cytokine secretion by garlic oil derivatives is associated with suppressed nitric oxide production in stimulated macrophages. *J Agric Food Chem* 2005;**53**:2530–2534.
- Cheeseman KH, Slater TF. An introduction to free radical biochemistry. *Br Med Bull* 1993;**49**:481–493.

- Chen J, Siddiqui A. Hepatitis B virus X protein stimulates the mitochondrial translocation of Raf-1 via oxidative stress. *J Virol* 2007;**81**:6757–6760.
- Chen SS, Huang WJ, Chang LS, Wei YH. 8-hydroxy-2'-deoxyguanosine in leukocyte DNA of spermatic vein as a biomarker of oxidative stress in patients with varicocele. *J Urol* 2004;**172**:1418–1421.
- Chitra KC, Sujatha R, Latchoumycandane C, Mathur PP. Effect of lindane on antioxidant enzymes in epididymis and epididymal sperm of adult rats. *Asian J Androl* 2001;**3**:205–208.
- Christiansen E, Tollefsrud A, Purvis K. Sperm quality in men with chronic abacterial prostatovesiculitis verified by rectal ultrasonography. *Urology* 1991;**38**:545–549.
- Comhaire FH, Christophe AB, Zalata AA, Dhooge WS, Mahmoud AM, Depuydt CE. The effects of combined conventional treatment, oral antioxidants and essential fatty acids on sperm biology in subfertile men. *Prostaglandins Leukot Essent Fatty Acids* 2000;**63**:159–165.
- Comhaire FH, El Garem Y, Mahmoud A, Eertmans F, Schoonjans F. Combined conventional/antioxidant 'Astaxanthin' treatment for male infertility: a double blind, randomized trial. *Asian J Androl* 2005;**7**:257–262.
- Dandekar SP, Nadkarni GD, Kulkarni VS, Puneekar S. Lipid peroxidation and antioxidant enzymes in male infertility. *J Postgrad Med* 2002;**48**:186–189. discussion 189–190.
- Danielski M, Ikizler TA, McMonagle E, Kane JC, Pupim L, Morrow J, Himmelfarb J. Linkage of hypoalbuminemia, inflammation, and oxidative stress in patients receiving maintenance hemodialysis therapy. *Am J Kidney Dis* 2003;**42**:286–294.
- Das UB, Mallick M, Debnath JM, Ghosh D. Protective effect of ascorbic acid on cyclophosphamide-induced testicular gametogenic and androgenic disorders in male rats. *Asian J Androl* 2002;**4**:201–207.
- de Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. II Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl* 1992;**13**:379–386.
- de Lamirande E, Gagnon C. Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radic Biol Med* 1993;**14**:157–166.
- de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. *Rev Reprod* 1997;**2**:48–54.
- de Lamirande E, Tsai C, Harakat A, Gagnon C. Involvement of reactive oxygen species in human sperm arcosome reaction induced by A23187, lysophosphatidylcholine, and biological fluid ultrafiltrates. *J Androl* 1998;**19**:585–594.
- Depuydt CE, Bosmans E, Zalata A, Schoonjans F, Comhaire FH. The relation between reactive oxygen species and cytokines in andrological patients with or without male accessory gland infection. *J Androl* 1996;**17**:699–707.
- Donnelly ET, McClure N, Lewis SE. The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis* 1999;**14**:505–512.
- Donnelly ET, McClure N, Lewis SE. Glutathione and hypotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species. *Mutagenesis* 2000;**15**:61–68.
- Durazzo M, Premoli A, Di Bisceglie C, Bertagna A, Faga E, Biroli G, Manieri C, Bo S, Pagano G. Alterations of seminal and hormonal parameters: an extrahepatic manifestation of HCV infection? *World J Gastroenterol* 2006;**12**:3073–3076.
- Engeler DS, Hauri D, John H. Impact of prostatitis NIH IIIB (prostatodynia) on ejaculate parameters. *Eur Urol* 2003;**44**:546–548.
- Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 2004;**37**:277–285.
- Esfandiari N, Sharma RK, Saleh RA, Thomas AJ, Jr, Agarwal A. Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. *J Androl* 2003;**24**:862–870.
- Eskenazi B, Kidd SA, Marks AR, Slotter E, Block G, Wyrobek AJ. Antioxidant intake is associated with semen quality in healthy men. *Hum Reprod* 2005;**20**:1006–1012.
- Eskioçak S, Gozen AS, Yapar SB, Tavas F, Kilic AS, Eskioçak M. Glutathione and free sulphhydryl content of seminal plasma in healthy medical students during and after exam stress. *Hum Reprod* 2005;**20**:2595–2600.
- Eskioçak S, Gozen AS, Taskiran A, Kilic AS, Eskioçak M, Gulen S. Effect of psychological stress on the L-arginine-nitric oxide pathway and semen quality. *Braz J Med Biol Res* 2006;**39**:581–588.
- Evers JL, Collins JA. Surgery or embolisation for varicocele in subfertile men. *Cochrane Database Syst Rev* 2004; CD000479.
- Fenster L, Katz DF, Wyrobek AJ, Pieper C, Rempel DM, Oman D, Swan SH. Effects of psychological stress on human semen quality. *J Androl* 1997;**18**:194–202.
- Filho DW, Torres MA, Bordin AL, Crezcynski-Pasa TB, Boveris A. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury. *Mol Aspects Med* 2004;**25**:199–210.
- Filippini A, Riccioli A, Padula F, Lauretti P, D'Alessio A, De Cesaris P, Gandini L, Lenzi A, Ziparo E. Control and impairment of immune privilege in the testis and in semen. *Hum Reprod Update* 2001;**7**:444–449.
- Fisher H, Aitken R. Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. *J Exp Zool* 1997;**277**:390–400.
- Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. Impact of folate and homocysteine metabolism on human reproductive health. *Hum Reprod Update* 2007;**13**:225–238.
- Fraczek M, Kurpisz M. Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa. *J Androl* 2007;**28**:325–333.
- Fraczek M, Sanocka D, Kamieniczna M, Kurpisz M. Proinflammatory cytokines as an intermediate factor enhancing lipid sperm membrane peroxidation in vitro conditions. *J Androl* 2008;**29**:85–92.
- Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci USA* 1991;**88**:11003–11006.
- Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* 1996;**351**:199–203.
- Fujii J, Iuchi Y, Matsuki S, Ishii T. Cooperative function of antioxidant and redox systems against oxidative stress in male reproductive tissues. *Asian J Androl* 2003;**5**:231–242.
- Gambera L, Serafini F, Morgante G, Focarelli R, De Leo V, Piomboni P. Sperm quality and pregnancy rate after COX-2 inhibitor therapy of infertile males with abacterial leukospermia. *Hum Reprod* 2007;**22**:1047–1051.
- Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J. Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. *Asian J Androl* 2004a;**6**:59–65.
- Garrido N, Meseguer M, Alvarez J, Simon C, Pellicer A, Remohi J. Relationship among standard semen parameters, glutathione peroxidase/glutathione reductase activity, and mRNA expression and reduced glutathione content in ejaculated spermatozoa from fertile and infertile men. *Fertil Steril* 2004b;**82**(Suppl 3):1059–1066.
- Gennart JP, Buchet JP, Roels H, Ghyselen P, Ceulemans E, Lauwerys R. Fertility of male workers exposed to cadmium, lead, or manganese. *Am J Epidemiol* 1992;**135**:1208–1219.
- Ghosh D, Das UB, Misro M. Protective role of alpha-tocopherol-succinate (provitamin-E) in cyclophosphamide induced testicular gametogenic and steroidogenic disorders: a correlative approach to oxidative stress. *Free Radic Res* 2002;**36**:1209–1218.
- Giannattasio A, De Rosa M, Smeraglia R, Zarrilli S, Cimmino A, Di Rosario B, Ruggiero R, Colao A, Lombardi G. Glutathione peroxidase (GPX) activity in seminal plasma of healthy and infertile males. *J Endocrinol Invest* 2002;**25**:983–986.
- Gomez E, Aitken J. Impact of in vitro fertilization culture media on peroxidative damage to human spermatozoa. *Fertil Steril* 1996;**65**:880–882.
- Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 1996;**17**:276–287.
- Gonzalez-Flecha B. Oxidant mechanisms in response to ambient air particles. *Mol Aspects Med* 2004;**25**:169–182.
- Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* 2005a;**26**:349–353.
- Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Franco G, Anniballo N, Mendoza C, Tesarik J. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Hum Reprod* 2005b;**20**:226–230.
- Griveau JF, Le Lannou D. Influence of oxygen tension on reactive oxygen species production and human sperm function. *Int J Androl* 1997;**20**:195–200.
- Guha M, Kumar S, Choubey V, Maity P, Bandyopadhyay U. Apoptosis in liver during malaria: role of oxidative stress and implication of mitochondrial pathway. *FASEB J* 2006;**20**:1224–1226.

- Gurbuz B, Yalti S, Ficicioglu C, Zehir K. Relationship between semen quality and seminal plasma total carnitine in infertile men. *J Obstet Gynaecol* 2003;**23**:653–656.
- Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S, Calafat AM. DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Hum Reprod* 2007;**22**:688–695.
- Hendin BN, Kolettis PN, Sharma RK, Thomas AJ, Jr, Agarwal A. Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Urol* 1999;**161**:1831–1834.
- Henkel R, Kierspelt E, Stalf T, Mehnert C, Menkveld R, Tinneberg HR, Schill WB, Kruger TF. Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. *Fertil Steril* 2005;**83**:635–642.
- Henkel R, Ludwig M, Schuppe HC, Diemer T, Schill WB, Weidner W. Chronic pelvic pain syndrome/chronic prostatitis affect the acrosome reaction in human spermatozoa. *World J Urol* 2006;**24**:39–44.
- Hodge G, Hodge S, Han P. Allium sativum (garlic) suppresses leukocyte inflammatory cytokine production in vitro: potential therapeutic use in the treatment of inflammatory bowel disease. *Cytometry* 2002;**48**:209–215.
- Hsieh YY, Sun YL, Chang CC, Lee YS, Tsai HD, Lin CS. Superoxide dismutase activities of spermatozoa and seminal plasma are not correlated with male infertility. *J Clin Lab Anal* 2002;**16**:127–131.
- Hsieh YY, Chang CC, Lin CS. Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. *Int J Biol Sci* 2006;**2**:23–29.
- Hsu PC, Guo YL. Antioxidant nutrients and lead toxicity. *Toxicology* 2002;**180**:33–44.
- Huff DS, Hadziselimovic F, Snyder HM, 3rd, Blyth B, Duckett JW. Early postnatal testicular maldevelopment in cryptorchidism. *J Urol* 1991;**146**:624–626.
- Hurtado de Catalfo GE, Ranieri-Casilla A, Marra FA, de Alaniz MJ, Marra CA. Oxidative stress biomarkers and hormonal profile in human patients undergoing varicocele. *Int J Androl* 2007;**30**:519–530.
- Ichikawa T, Oeda T, Ohmori H, Schill WB. Reactive oxygen species influence the acrosome reaction but not acrosin activity in human spermatozoa. *Int J Androl* 1999;**22**:37–42.
- Ishii T, Matsuki S, Iuchi Y, Okada F, Toyosaki S, Tomita Y, Ikeda Y, Fujii J. Accelerated impairment of spermatogenic cells in SOD1-knockout mice under heat stress. *Free Radic Res* 2005;**39**:697–705.
- Ishikawa T, Fujioka H, Ishimura T, Takenake A, Fujisawa M. Increased testicular 8-hydroxy-2'-deoxyguanosine in patients with varicocele. *BJU Int* 2007;**100**:863–866.
- Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992;**57**:409–416.
- Jedrejczak P, Fraczek M, Szumala-Kakol A, Taszarek-Hauke G, Pawelczyk L, Kurpiz M. Consequences of semen inflammation and lipid peroxidation on fertilization capacity of spermatozoa in vitro conditions. *Int J Androl* 2005;**28**:275–283.
- Jeulin C, Soufir JC, Weber P, Laval-Martin D, Calvayrac R. Catalase activity in human spermatozoa and seminal plasma. *Gamete Res* 1989;**24**:185–196.
- Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fert Steril* 1979;**31**:531–537.
- Junqueira VB, Barros SB, Chan SS, Rodrigues L, Giavarotti L, Abud RL, Deucher GP. Aging and oxidative stress. *Mol Aspects Med* 2004;**25**:5–16.
- Kao SH, Chao HT, Chen HW, Hwang TI, Liao TL, Wei YH. Increase of oxidative stress in human sperm with lower motility. *Fertil Steril* 2007. (30 July, 2007, online publication ahead of print).
- Kapranos N, Petrakou E, Anastasiadou C, Kotronias D. Detection of herpes simplex virus, cytomegalovirus, and Epstein-Barr virus in the semen of men attending an infertility clinic. *Fertil Steril* 2003;**79**(Suppl):1566–1570.
- Kasahara E, Sato EF, Miyoshi M, Konaka R, Hiramoto K, Sasaki J, Tokuda M, Nakano Y, Inoue M. Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. *Biochem J* 2002;**365**:849–856.
- Kemal Duru N, Morshedi M, Oehninger S. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. *Fertil Steril* 2000;**74**:1200–1207.
- Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghazzi H, Hammami S, Zghal K, Fki H, Damak J, Bahloul A. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Arch Androl* 2003;**49**:83–94.
- Kessopoulou E, Powers HJ, Sharma KK, Pearson MJ, Russell JM, Cooke ID, Barratt CL. A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertil Steril* 1995;**64**:825–831.
- Khosrowbeygi A, Zarghami N. Levels of oxidative stress biomarkers in seminal plasma and their relationship with seminal parameters. *BMC Clin Pathol* 2007;**7**:6.
- Kobayashi T, Miyazaki T, Natori M, Nozawa S. Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Hum Reprod* 1991;**6**:987–991.
- Kobayashi H, Gil-Guzman E, Mahran AM, Rakesh, Nelson DR, Thomas AJ, Jr, Agarwa A. Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. *J Androl* 2001;**22**:568–574.
- Koca Y, Ozdal OL, Celik M, Unal S, Balaban N. Antioxidant activity of seminal plasma in fertile and infertile men. *Arch Androl* 2003;**49**:355–359.
- Koch OR, Pani G, Borrello S, Colavitti R, Cravero A, Farre S, Galeotti T. Oxidative stress and antioxidant defenses in ethanol-induced cell injury. *Mol Aspects Med* 2004;**25**:191–198.
- Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997;**68**:519–524.
- Kolettis PN, Sharma RK, Pasqualotto FF, Nelson D, Thomas AJ, Jr, Agarwal A. Effect of seminal oxidative stress on fertility after vasectomy reversal. *Fertil Steril* 1999;**71**:249–255.
- Kopa Z, Wenzel J, Papp GK, Haidl G. Role of granulocyte elastase and interleukin-6 in the diagnosis of male genital tract inflammation. *Andrologia* 2005;**37**:188–194.
- Krause W, Herbstreit F, Slenzka W. Are viral infections the cause of leukocytospermia? *Andrologia* 2002;**34**:87–90.
- Krause W, Bohring C, Gueth A, Horster S, Krisp A, Skrzypek J. Cellular and biochemical markers in semen indicating male accessory gland inflammation. *Andrologia* 2003;**35**:279–282.
- Krieger JN, Berger RE, Ross SO, Rothman I, Muller CH. Seminal fluid findings in men with nonbacterial prostatitis and prostatodynia. *J Androl* 1996;**17**:310–318.
- Kvist U, Bjorn Dahl L, Kjellberg S. Sperm nuclear zinc, chromatin stability, and male fertility. *Scanning Microsc* 1987;**1**:1241–1247.
- Latchoumycandane C, Mathur PP. Induction of oxidative stress in the rat testis after short-term exposure to the organochlorine pesticide methoxychlor. *Arch Toxicol* 2002;**76**:692–698.
- Latchoumycandane C, Chitra KC, Mathur PP. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces oxidative stress in the epididymis and epididymal sperm of adult rats. *Arch Toxicol* 2003;**77**:280–284.
- Lee HC, Jeong YM, Lee SH, Cha KY, Song SH, Kim NK, Lee KW, Lee S. Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility. *Hum Reprod* 2006;**21**:3162–3170.
- Lee E, Ahn MY, Kim HJ, Kim IY, Han SY, Kang TS, Hong JH, Park KL, Lee BM, Kim HS. Effect of di(n-butyl) phthalate on testicular oxidative damage and antioxidant enzymes in hyperthyroid rats. *Environ Toxicol* 2007;**22**:245–255.
- Leib Z, Bartoov B, Eltes F, Servadio C. Reduced semen quality caused by chronic abacterial prostatitis: an enigma or reality? *Fertil Steril* 1994;**61**:1109–1116.
- Lenzi A, Culasso F, Gandini L, Lombardo F, Dondero F. Placebo-controlled, double-blind, cross-over trial of glutathione therapy in male infertility. *Hum Reprod* 1993;**8**:1657–1662.
- Lenzi A, Sgro P, Salacone P, Paoli D, Gilio B, Lombardo F, Santulli M, Agarwal A, Gandini L. A placebo-controlled double-blind randomized trial of the use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. *Fertil Steril* 2004;**81**:1578–1584.
- Lewis SE, Sterling ES, Young IS, Thompson W. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertil Steril* 1997;**67**:142–147.
- Li K, Shang X, Chen Y. High-performance liquid chromatographic detection of lipid peroxidation in human seminal plasma and its application to male infertility. *Clin Chim Acta* 2004;**346**:199–203.
- Liochev SI, Fridovich I. Lucigenin (Bis-N-methylacridinium) as a mediator of superoxide anion production. *Archives Biochem Biophys* 1997;**337**:115–120.
- Livrea MA, Tesoriere L, Pintaudi AM, Calabrese A, Maggio A, Freisleben HJ, D'Arpa D, D'Anna R, Bongiorno A. Oxidative stress and antioxidant status in beta-thalassemia major: iron overload and depletion of lipid-soluble antioxidants. *Blood* 1996;**88**:3608–3614.

- Loft S, Kold-Jensen T, Hjollund NH, Giwercman A, Gylleborg J, Ernst E, Olsen J, Scheike T, Poulsen HE, Bonde JP. Oxidative DNA damage in human sperm influences time to pregnancy. *Hum Reprod* 2003;**18**:1265–1272.
- Ludwig M, Vidal A, Huwe P, Diemer T, Pabst W, Weidner W. Significance of inflammation on standard semen analysis in chronic prostatitis/chronic pelvic pain syndrome. *Andrologia* 2003;**35**:152–156.
- Macao LB, Filho DW, Pedrosa RC, Pereira A, Backes P, Torres MA, Frode TS. Antioxidant therapy attenuates oxidative stress in chronic cardiopathy associated with Chagas' disease. *Int J Cardiol* 2007;**123**:43–49.
- MacLeod J. The role of oxygen in the metabolism and motility of human spermatozoa. *Am J Physiol* 1943;**138**:512–518.
- Maneesh M, Dutta S, Chakrabarti A, Vasudevan DM. Alcohol abuse-duration dependent decrease in plasma testosterone and antioxidants in males. *Indian J Physiol Pharmacol* 2006;**50**:291–296.
- Manna I, Jana K, Samanta PK. Effect of different intensities of swimming exercise on testicular oxidative stress and reproductive dysfunction in mature male albino Wistar rats. *Indian J Exp Biol* 2004;**42**:816–822.
- Marmar JL, Agarwal A, Prabakaran S, Agarwal R, Short RA, Benoff S, Thomas AJ, Jr. Reassessing the value of varicocelectomy as a treatment for male subfertility with a new meta-analysis. *Fertil Steril* 2007;**88**:639–648.
- Martinez P, Proverbio F, Camejo MI. Sperm lipid peroxidation and pro-inflammatory cytokines. *Asian J Androl* 2007;**9**:102–107.
- Matthews RG. Methylene-tetrahydrofolate reductase: a common human polymorphism and its biochemical implications. *Chem Rec* 2002;**2**:4–12.
- Mazzilli F, Rossi T, Marchesini M, Ronconi C, Dondero F. Superoxide anion in human semen related to seminal parameters and clinical aspects. *Fertil Steril* 1994;**62**:862–868.
- McLachlan R, de Kretser D. Male infertility: The case for continued research. *MJA* 2001;**174**:116–117.
- Menezo YJ, Hazout A, Panteix G, Robert F, Rollet J, Cohen-Bacrie P, Chapuis F, Clement P, Benkhalifa M. Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. *Reprod Biomed Online* 2007;**14**:418–421.
- Meng Z, Bai W. Oxidation damage of sulfur dioxide on testicles of mice. *Environ Res* 2004;**96**:298–304.
- Mennella M, Jones R. Properties of spermatozoal superoxide dismutase and lack of involvement of superoxides in metal-ion-catalysed lipid-peroxidation and reactions in semen. *Biochem J* 1980;**191**:289–297.
- Meseguer M, de los Santos MJ, Simon C, Pellicer A, Remohi J, Garrido N. Effect of sperm glutathione peroxidases 1 and 4 on embryo asymmetry and blastocyst quality in oocyte donation cycles. *Fertil Steril* 2006;**86**:1376–1385.
- Meseguer M, Martinez-Conejero JA, O'Connor JE, Pellicer A, Remohi J, Garrido N. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. *Fertil Steril* 2007 (4 August, 2007, online publication ahead of print).
- Miesel R, Jedrzejczak P, Sanocka D, Kurpisz MK. Severe antioxidant deficiency in human semen samples with pathological spermogram parameters. *Andrologia* 1997;**29**:77–83.
- Moreno JM, Ruiz MC, Ruiz N, Gomez I, Vargas F, Asensio C, Osuna A. Modulation factors of oxidative status in stable renal transplantation. *Transplant Proc* 2005;**37**:1428–1430.
- Moskovtsev SI, Willis J, Mullen JB. Age-related decline in sperm deoxyribonucleic acid integrity in patients evaluated for male infertility. *Fertil Steril* 2006;**85**:496–499.
- Mostafa T, Anis TH, El-Nashar A, Imam H, Othman IA. Varicocelectomy reduces reactive oxygen species levels and increases antioxidant activity of seminal plasma from infertile men with varicocele. *Int J Androl* 2001;**24**:261–265.
- Mostafa T, Tawadrous G, Roaia MM, Amer MK, Kader RA, Aziz A. Effect of smoking on seminal plasma ascorbic acid in infertile and fertile males. *Andrologia* 2006;**38**:221–224.
- Motrich RD, Maccioni M, Molina R, Tissera A, Olmedo J, Riera CM, Rivero VE. Reduced semen quality in chronic prostatitis patients that have cellular autoimmune response to prostate antigens. *Hum Reprod* 2005;**20**:2567–2572.
- Motrich RD, Maccioni M, Riera CM, Rivero VE. Autoimmune prostatitis: state of the art. *Scand J Immunol* 2007;**66**:217–227.
- Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ, Jr, Agarwal A. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 2004;**19**:129–138.
- Naha N, Chowdhury AR. Inorganic lead exposure in battery and paint factory: effect on human sperm structure and functional activity. *J UOEH* 2006;**28**:157–171.
- Nakamura H, Kimura T, Nakajima A, Shimoya K, Takemura M, Hashimoto K, Isaka S, Azuma C, Koyama M, Murata Y. Detection of oxidative stress in seminal plasma and fractionated sperm from subfertile male patients. *Eur J Obstet Gynecol Reprod Biol* 2002;**105**:155–160.
- Nallella KP, Allamaneni SS, Pasqualotto FF, Sharma RK, Thomas AJ, Jr, Agarwal A. Relationship of interleukin-6 with semen characteristics and oxidative stress in patients with varicocele. *Urology* 2004;**64**:1010–1013.
- Nandipati KC, Pasqualotto FF, Thomas AJ, Jr, Agarwal A. Relationship of interleukin-6 with semen characteristics and oxidative stress in vasectomy reversal patients. *Andrologia* 2005;**37**:131–134.
- Oberg BP, McMenamin E, Lucas FL, McMonagle E, Morrow J, Ikizler TA, Himmelfarb J. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 2004;**65**:1009–1016.
- Ochsendorf FR. Infections in the male genital tract and reactive oxygen species. *Hum Reprod Update* 1999;**5**:399–420.
- Ochsendorf FR, Thiele J, Fuchs J, Schuttat H, Freisleben HJ, Buslau M, Milbradt R. Chemiluminescence in semen of infertile men. *Andrologia* 1994;**26**:289–293.
- O'Connell M, McClure N, Lewis SE. Mitochondrial DNA deletions and nuclear DNA fragmentation in testicular and epididymal human sperm. *Hum Reprod* 2002;**17**:1565–1570.
- Oeda T, Henkel R, Ohmori H, Schill WB. Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility? *Andrologia* 1997;**29**:125–131.
- Oger I, Da Cruz C, Panteix G, Menezo Y. Evaluating human sperm DNA integrity: relationship between 8-hydroxydeoxyguanosine quantification and the sperm chromatin structure assay. *Zygote* 2003;**11**:367–371.
- Oliva R. Protamines and male infertility. *Hum Reprod Update* 2006;**12**:417–435.
- Omu AE, al-Othman S, Mohamad AS, al-Kaluwby NM, Fernandes S. Antibiotic therapy for seminal infection. Effect on antioxidant activity and T-helper cytokines. *J Reprod Med* 1998;**43**:857–864.
- Ozmen B, Koutlaki N, Youssry M, Diedrich K, Al-Hasani S. DNA damage of human spermatozoa in assisted reproduction: origins, diagnosis, impacts and safety. *Reprod Biomed Online* 2007;**14**:384–395.
- Padron OF, Brackett NL, Sharma RK, Lynne CM, Thomas AJ, Jr, Agarwal A. Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. *Fertil Steril* 1997;**67**:1115–1120.
- Park JH, Lee HC, Jeong YM, Chung TG, Kim HJ, Kim NK, Lee SH, Lee S. MTHFR C677T polymorphism associates with unexplained infertile male factors. *J Assist Reprod Genet* 2005;**22**:361–368.
- Pasqualotto FF, Sharma RK, Potts JM, Nelson DR, Thomas AJ, Agarwal A. Seminal oxidative stress in patients with chronic prostatitis. *Urology* 2000;**55**:881–885.
- Pasqualotto FF, Sharma RK, Kobayashi H, Nelson DR, Thomas AJ, Jr, Agarwal A. Oxidative stress in normospermic men undergoing infertility evaluation. *J Androl* 2001;**22**:316–322.
- Peake JM, Suzuki K, Coombes JS. The influence of antioxidant supplementation on markers of inflammation and the relationship to oxidative stress after exercise. *J Nutr Biochem* 2007;**18**:357–371.
- Perez-Crespo M, Pintado B, Gutierrez-Adan A. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev* 2007;**75**:40–47.
- Pfeifer H, Conrad M, Roethlein D, Kyriakopoulos A, Brielmeier M, Bornkamm GW, Behne D. Identification of a specific sperm nuclei selenoenzyme necessary for protamine thiol cross-linking during sperm maturation. *FASEB J* 2001;**15**:1236–1238.
- Plante M, de Lamirande E, Gagnon C. Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. *Fertil Steril* 1994;**62**:387–393.
- Potts JM, Pasqualotto FF. Seminal oxidative stress in patients with chronic prostatitis. *Andrologia* 2003;**35**:304–308.
- Potts JM, Sharma R, Pasqualotto F, Nelson D, Hall G, Agarwal A. Association of *Ureaplasma urealyticum* with abnormal reactive oxygen species levels and absence of leukocytospermia. *J Urol* 2000a;**163**:1775–1778.
- Potts RJ, Notarianni LJ, Jefferies TM. Seminal plasma reduces exogenous oxidative damage to human sperm, determined by the measurement of

- DNA strand breaks and lipid peroxidation. *Mutat Res* 2000b;447:249–256.
- Pupim LB, Himmelfarb J, McMonagle E, Shyr Y, Ikizler TA. Influence of initiation of maintenance hemodialysis on biomarkers of inflammation and oxidative stress. *Kidney Int* 2004;65:2371–2379.
- Rajasekaran M, Hellstrom WJ, Naz RK, Sikka SC. Oxidative stress and interleukins in seminal plasma during leukocytospermia. *Fertil Steril* 1995;64:166–171.
- Richer SC, Ford WCL. A critical investigation of NADPH oxidase activity in human spermatozoa. *Mol Hum Reprod* 2001;7:237–244.
- Rodin DM, Larone D, Goldstein M. Relationship between semen cultures, leukospermia and semen analysis in men undergoing fertility evaluation. *Fertil Steril* 2003;79:1555–1558.
- Rolf C, Cooper TG, Yeung CH, Nieschlag E. Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: a randomized, placebo-controlled, double-blind study. *Hum Reprod* 1999;14:1028–1033.
- Rossi T, Mazzilli F, Delfino M, Dondero F. Improved human sperm recovery using superoxide dismutase and catalase supplementation in semen cryopreservation procedure. *Cell Tissue Bank* 2001;2:9–13.
- Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, Robbins WA, Perreault SD. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. *Hum Reprod* 2005;20:2776–2783.
- Sabeur K, Ball BA. Characterization of NADPH oxidase 5 in equine testis and spermatozoa. *Reproduction* 2007;134:263–270.
- Saez F, Motta C, Boucher D, Grizard G. Antioxidant capacity of prostasomes in human semen. *Mol Hum Reprod* 1998;4:667–672.
- Said TM, Kattal N, Sharma RK, Sikka SC, Thomas AJ, Jr, Mascha E, Agarwal A. Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. *J Androl* 2003;24:676–680.
- Said TM, Agarwal A, Sharma RK, Mascha E, Sikka SC, Thomas AJ, Jr. Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. *Fertil Steril* 2004;82:871–877.
- Said TM, Agarwal A, Sharma RK, Thomas AJ, Jr, Sikka SC. Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. *Fertil Steril* 2005;83:95–103.
- Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ, Jr. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. *Fertil Steril* 2002a;78:491–499.
- Saleh RA, Agarwal A, Kandirali E, Sharma RK, Thomas AJ, Nada EA, Evenson DP, Alvarez JG. Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertil Steril* 2002b;78:1215–1224.
- Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR, Thomas AJ. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003a;79(Suppl 3):1597–1605.
- Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, Thomas AJ. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. *Fertil Steril* 2003b;80:1431–1436.
- Sanocka D, Miesel R, Jedrzejczak P, Chelmonska-Soyta AC, Kurpisz M. Effect of reactive oxygen species and the activity of antioxidant systems on human semen; association with male infertility. *Int J Androl* 1997;20:255–264.
- Sanocka D, Jedrzejczak P, Szumala-Kaekol A, Fraczek M, Kurpisz M. Male genital tract inflammation: The role of selected interleukins in regulation of pro-oxidant and antioxidant enzymatic substances in seminal plasma. *J Androl* 2003;24:448–455.
- Schaeffer AJ. Epidemiology and demographics of prostatitis. *Andrologia* 2003;35:252–257.
- Scott R, MacPherson A, Yates RW, Hussain B, Dixon J. The effect of oral selenium supplementation on human sperm motility. *Br J Urol* 1998;82:76–80.
- Segnini A, Camejo MI, Proverbio F. *Chlamydia trachomatis* and sperm lipid peroxidation in infertile men. *Asian J Androl* 2003;5:47–49.
- Selhub J. Homocysteine metabolism. *Annu Rev Nutr* 1999;19:217–246.
- Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril* 2004;82:378–383.
- Seronello S, Sheikh MY, Choi J. Redox regulation of hepatitis C in nonalcoholic and alcoholic liver. *Free Radic Biol Med* 2007;43:869–882.
- Shahed AR, Shoskes DA. Oxidative stress in prostatic fluid of patients with chronic pelvic pain syndrome: correlation with gram positive bacterial growth and treatment response. *J Androl* 2000;21:669–675.
- Shang XJ, Li K, Ye ZQ, Chen YG, Yu X, Huang YF. Analysis of lipid peroxidative levels in seminal plasma of infertile men by high-performance liquid chromatography. *Arch Androl* 2004;50:411–416.
- Shapiro RH, Muller CH, Chen G, Berger RE. Vasectomy reversal associated with increased reactive oxygen species production by seminal fluid leukocytes and sperm. *J Urol* 1998;160:1341–1346.
- Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ, Jr, Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum Reprod* 1999;14:2801–2807.
- Sharma RK, Pasqualotto AE, Nelson DR, Thomas AJ, Jr, Agarwal A. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl* 2001;22:575–583.
- Shekarriz M, Thomas AJ, Jr, Agarwal A. Incidence and level of seminal reactive oxygen species in normal men. *Urology* 1995a;45:103–107.
- Shekarriz M, DeWire DM, Thomas AJ, Jr, Agarwal A. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Urol* 1995b;28:31–35.
- Shoskes DA, Albakri Q, Thomas K, Cook D. Cytokine polymorphisms in men with chronic prostatitis/chronic pelvic pain syndrome: association with diagnosis and treatment response. *J Urol* 2002;168:331–335.
- Shrilatha B, Muralidhara DR. Occurrence of oxidative impairments, response of antioxidant defences and associated biochemical perturbations in male reproductive milieu in the Streptozotocin-diabetic rat. *Int J Androl* 2007;30:508–518.
- Siciliano L, Tarantino P, Longobardi F, Rago V, De Stefano C, Carpino A. Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligoasthenozoospermia. *J Androl* 2001;22:798–803.
- Silver EW, Eskenazi B, Evenson DP, Block G, Young S, Wyrobek AJ. Effect of antioxidant intake on sperm chromatin stability in healthy nonsmoking men. *J Androl* 2005;26:550–556.
- Singer G, Granger DN. Inflammatory responses underlying the microvascular dysfunction associated with obesity and insulin resistance. *Microcirculation* 2007;14:375–387.
- Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. *Fertil Steril* 2003;80:1420–1430.
- Skibinski G, Kelly RW, Harkiss D, James K. Immunosuppression by human seminal plasma-extracellular organelles (prostasomes) modulate activity of phagocytic cells. *Am J Reprod Immunol* 1992;28:97–103.
- Smith R, Vantman D, Ponce J, Escobar J, Lissi E. Total antioxidant capacity of human seminal plasma. *Hum Reprod* 1996;11:1655–1660.
- Smith R, Kaune H, Parodi D, Madariaga M, Rios R, Morales I, Castro A. Increased sperm DNA damage in patients with varicocele: relationship with seminal oxidative stress. *Hum Reprod* 2006;21:986–993.
- Smith GR, Kaune GH, Parodi Ch D, Madariaga AM, Morales DI, Rios SR, Castro GA. Extent of sperm DNA damage in spermatozoa from men examined for infertility. Relationship with oxidative stress. *Rev Med Chil* 2007;135:279–286.
- Song GJ, Norkus EP, Lewis V. Relationship between seminal ascorbic acid and sperm DNA integrity in infertile men. *Int J Androl* 2006;29:569–575.
- Sonmez M, Yuce A, Turk G. The protective effects of melatonin and Vitamin E on antioxidant enzyme activities and epididymal sperm characteristics of homocysteine treated male rats. *Reprod Toxicol* 2007;23:226–231.
- Srinivasan S, Pragasam V, Jenita X, Kalaiselvi P, Muthu V, Varalakshmi P. Oxidative stress in urogenital tuberculosis patients: a predisposing factor for renal stone formation—amelioration by vitamin E supplementation. *Clin Chim Acta* 2004;350:57–63.
- Srivastava SP, Srivastava S, Saxena DK, Chandra SV, Seth PK. Testicular effects of di-n-butyl phthalate (DBP): biochemical and histopathological alterations. *Arch Toxicol* 1990;64:148–152.
- Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 1996;17:530–537.
- Tavilani H, Doosti M, Saeidi H. Malondialdehyde levels in sperm and seminal plasma of asthenozoospermic and its relationship with semen parameters. *Clin Chim Acta* 2005;356:199–203.
- Therond P, Auger J, Legrand A, Jouannet P. alpha-Tocopherol in human spermatozoa and seminal plasma: relationships with motility, antioxidant enzymes and leukocytes. *Mol Hum Reprod* 1996;2:739–744.
- Thonneau P, Marchand S, Tallec A, Ferial ML, Ducot B, Lansac J, Lopes P, Tabaste JM, Spira A. Incidence and main causes of infertility in a

- resident population (1,850,000) of three French regions (1988-1989). *Hum Reprod* 1991;**6**:811–816.
- Tomlinson MJ, Barratt CL, Cooke ID. Prospective study of leukocytes and leukocyte subpopulations in semen suggests they are not a cause of male infertility. *Fertil Steril* 1993;**60**:1069–1075.
- Tremellen K, Miari G, Froiland D, Thompson J. A randomised control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. *Aust N Z J Obstet Gynaecol* 2007;**47**:216–221.
- Turner TT, Bang HJ, Lysiak JL. The molecular pathology of experimental testicular torsion suggests adjunct therapy to surgical repair. *J Urol* 2004;**172**:2574–2578.
- Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod* 1998;**13**:1429–1436.
- Umapathy E, Simbini T, Chipata T, Mbizvo M. Sperm characteristics and accessory sex gland functions in HIV-infected men. *Arch Androl* 2001;**46**:153–158.
- Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;**39**:44–84.
- Vernet P, Aitken RJ, Drevet JR. Antioxidant strategies in the epididymis. *Mol Cell Endocrinol* 2004;**216**:31–39.
- Vicari E. Effectiveness and limits of antimicrobial treatment on seminal leukocyte concentration and related reactive oxygen species production in patients with male accessory gland infection. *Hum Reprod* 2000;**15**:2536–2544.
- Vicari E, Calogero AE. Effects of treatment with carnitines in infertile patients with prostatic-vesiculo-epididymitis. *Hum Reprod* 2001;**16**:2338–2342.
- Vicari E, La Vignera S, Calogero AE. Antioxidant treatment with carnitines is effective in infertile patients with prostatovesiculoeididymitis and elevated seminal leukocyte concentrations after treatment with nonsteroidal anti-inflammatory compounds. *Fertil Steril* 2002;**78**:1203–1208.
- Vicari E, Arcoria D, Di Mauro C, Noto R, Noto Z, La Vignera S. Sperm output in patients with primary infertility and hepatitis B or C virus; negative influence of HBV infection during concomitant varicocele. *Minerva Med* 2006;**97**:65–77.
- Vijayaraghavan R, Suribabu CS, Sekar B, Oommen PK, Kavithalakshmi SN, Madhusudhanan N, Panneerselvam C. Protective role of vitamin E on the oxidative stress in Hansen's disease (Leprosy) patients. *Eur J Clin Nutr* 2005;**59**:1121–1128.
- Villegas J, Schulz M, Soto L, Iglesias T, Miska W, Sanchez R. Influence of reactive oxygen species produced by activated leukocytes at the level of apoptosis in mature human spermatozoa. *Fertil Steril* 2005;**83**:808–810.
- Wang X, Sharma RK, Sikka SC, Thomas AJ, Jr, Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril* 2003;**80**:531–535.
- Wang Y, Liang CL, Wu JQ, Xu C, Qin SX, Gao ES. Do *Ureaplasma urealyticum* infections in the genital tract affect semen quality? *Asian J Androl* 2006;**8**:562–568.
- Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 2000;**60–61**:481–492.
- Weir CP, Robaire B. Spermatozoa have decreased antioxidant enzymatic capacity and increased reactive oxygen species production during aging in the Brown Norway rat. *J Androl* 2007;**28**:229–240.
- Werthman P, Wixon R, Kasperson K, Evenson DP. Significant decrease in sperm deoxyribonucleic acid fragmentation after varicocelectomy. *Fertil Steril* 2007 (9 June, 2007, online publication ahead of print).
- Whittington K, Ford WC. The effect of incubation periods under 95% oxygen on the stimulated acrosome reaction and motility of human spermatozoa. *Mol Hum Reprod* 1998;**4**:1053–1057.
- Whittington K, Harrison SC, Williams KM, Day JL, McLaughlin EA, Hull MG, Ford WC. Reactive oxygen species (ROS) production and the outcome of diagnostic tests of sperm function. *Int J Androl* 1999;**22**:236–242.
- Williams AC, Ford WC. Functional significance of the pentose phosphate pathway and glutathione reductase in the antioxidant defenses of human sperm. *Biol Reprod* 2004;**71**:1309–1316.
- Williams AC, Ford WC. Relationship between reactive oxygen species production and lipid peroxidation in human sperm suspensions and their association with sperm function. *Fertil Steril* 2005;**83**:929–936.
- Wolff H. The biological significance of white blood cells in semen. *Fertil Steril* 1995;**63**:1143–1157.
- World Health Organization. Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucous Interaction, 4th edn. New York: Cambridge University Press, 1999.
- Wu D, Cederbaum AI. Alcohol, oxidative stress, and free radical damage. *Alcohol Res Health* 2003;**27**:277–284.
- Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW, Glaser RL, Pearson FS, Evenson D. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proc Natl Acad Sci USA* 2006;**103**:9601–9606.
- Xu DX, Shen HM, Zhu QX, Chua L, Wang QN, Chia SE, Ong CN. The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. *Mutat Res* 2003;**534**:155–163.
- Yenilmez E, Yildirmis S, Yulug E, Aydin S, Tekelioglu Y, Erdem E, Topbas M, Arvas H. Ham's F-10 medium and Ham's F-10 medium plus vitamin E have protective effect against oxidative stress in human semen. *Urology* 2006;**67**:384–387.
- Zalata AA, Ahmed AH, Allamaneni SS, Comhaire FH, Agarwal A. Relationship between acrosin activity of human spermatozoa and oxidative stress. *Asian J Androl* 2004;**6**:313–318.
- Zheng RL, Zhang H. Effects of ferulic acid on fertile and asthenozoospermic infertile human sperm motility, viability, lipid peroxidation, and cyclic nucleotides. *Free Radic Biol Med* 1997;**22**:581–586.
- Zhou-Cun A, Yang Y, Zhang SZ, Li N, Zhang W. Single nucleotide polymorphism C677T in the methylenetetrahydrofolate reductase gene might be a genetic risk factor for infertility for Chinese men with azoospermia or severe oligozoospermia. *Asian J Androl* 2007;**9**:57–62.
- Zini A, de Lamirande E, Gagnon C. Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. *Int J Androl* 1993;**16**:183–188.
- Zini A, Garrels K, Phang D. Antioxidant activity in the semen of fertile and infertile men. *Urology* 2000;**55**:922–926.
- Zini A, Fischer MA, Nam RK, Jarvi K. Use of alternative and hormonal therapies in male infertility. *Urology* 2004;**63**:141–143.
- Zini A, Blumenfeld A, Libman J, Willis J. Beneficial effect of microsurgical varicocelectomy on human sperm DNA integrity. *Hum Reprod* 2005;**20**:1018–1021.
- Zorn B, Sesek-Briski A, Osredkar J, Meden-Vrtovec H. Semen polymorphonuclear neutrophil leukocyte elastase as a diagnostic and prognostic marker of genital tract inflammation—a review. *Clin Chem Lab Med* 2003b;**41**:2–12.
- Zorn B, Vidmar G, Meden-Vrtovec H. Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection. *Int J Androl* 2003a;**26**:279–285.
- Zubkova EV, Wade M, Robaire B. Changes in spermatozoal chromatin packaging and susceptibility to oxidative challenge during aging. *Fertil Steril* 2005;**84**(Suppl 2):1191–1198.

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