Review

Oxidative stress and medical antioxidant treatment in male infertility

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Abstract

Oxidative stress (OS) has been recognized as one of the most important cause of male infertility. Despite the antioxidant activity of seminal plasma, epididymis and spermatozoa, OS damages sperm function and DNA integrity. Since antioxidants suppress the action of reactive oxygen species, these compounds have been used in the medical treatment of male infertility or have been added to the culture medium during sperm separation techniques. Nevertheless, the efficacy of such a treatment has been reported to be very limited. This may relate to: (i) patient selection bias; (ii) late diagnosis of male infertility; (iii) lack of double-blind, placebo-controlled clinical trial; and/or (iv) use of end-points that are not good markers of the presence of OS. This review considers the effects of the main antioxidant compounds used in clinical practice. Overall, the data published suggest that no single antioxidant is able to enhance fertilizing capability in infertile men, whereas a combination of them seems to provide a better approach. Taking into account the pros and the cons of antioxidant treatment of male infertility, the potential advantages that it offers cannot be ignored. Therefore, antioxidant therapy should remain in the forefront of preventive medicine, including human reproductive medicine.

Keywords: *antioxidant treatment, male infertility, oxidative stress, spermatozoa*

Introduction

Widely accepted scientific evidence supports the role of oxidative stress (OS) as a causative factor in many human degenerative processes, diseases, syndromes and ageing processes (Cutler, 1991; Davies, 1995; Jacob and Burri, 1996; Cutler *et al.*, 2005). OS has been defined as an imbalance between the generation of reactive oxygen species (ROS) and antioxidant scavenging activities, in which the former prevails (Sikka, 2001).

In recent years, OS and the role of ROS in the pathophysiology of human sperm function and male infertility have been explored intensively. Indeed, spermatozoa, from the moment that they are produced in the testes to being ejaculated into the female reproductive tract, are constantly exposed to oxidizing environments. They are extremely sensitive to ROS because of their high content of polyunsaturated fatty acids (PUFA) and their limited ability to repair DNA (Griveau and Le Lannou, 1997; Shen and Ong, 2000).

Given the difficulty of reaching an accurate diagnosis, many antioxidant therapies have been used in the hope of improving sperm quality. Treatments have varied over the years involving the use of many different compounds, such as carnitines, phosphatidylcholine, kallikrein, pentoxifylline and vitamins A, E and C, without particular attention to counteracting the lipoperoxidative damage (Mann and Lutwak-Mann, 1981; Lanzafame *et al.*, 1994).

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The aim of this article is to review the pathways of sperm OS and antioxidant defences to better understand which conditions are at risk of disequilibrium, and which antioxidant therapies can lead to a real improvement of human sperm quality *in vitro* and *in vivo*.

Reactive oxygen species and spermatozoa

Although necessary for survival, oxygen also leads to production of free radicals. These are atomic or molecular species with unpaired electrons on an otherwise open-shell configuration. Unpaired electrons are usually highly reactive, so radicals are likely to take part in chemical reactions that damage sperm plasma membrane lipids (Jones and Mann, 1973, 1977), the so-called lipid peroxidation. This research area has received a great impulse and the importance of ROS generation and lipid peroxidation has been underlined as a mechanism that damages mammalian spermatozoa (Jones and Mann, 1973, 1977; Jones *et al.*, 1979; Saleh and Agarwal, 2002).

ROS are highly reactive oxidizing agents. These include the superoxide anion radical, the hydroxyl radical, the peroxyl radical and a subclass of free radicals derived from nitrogen, which includes nitric oxide, peroxynitrite, nitroxyl anion and peroxynitrous acid (**Table 1**). Although hydrogen peroxide, singlet oxygen and hydrochlorous acid should not be classified as free radicals because they still contain a pair of electrons in the outer orbital, often these are also included as oxyradical species (Forman and Boveris, 1984; Pryor, 1984; Warren *et al.*, 1987). The principal ROS produced by spermatozoa seems to be the superoxide anion radical, which generates hydrogen peroxide, spontaneously or following the activity of superoxide dismutase (SOD) (Alvarez *et al.*, 1987). In the microenvironment of cell membranes, hydrogen peroxide is the most stable intermediate of oxygen reduction (Aitken and Clarkson, 1987; Alvarez and Storey, 1989).

In contrast to the superoxide anion radical, hydrogen peroxide can effortlessly go through the plasma membrane and, despite its weak oxidizing capacity, if the scavenger function is inadequate to eliminate completely hydrogen peroxide and Fe or Cu is present, it promotes (by the Haber–Weiss reaction, $Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$, and the subsequent Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\cdot}$) the formation of hydroxyl radical, which is a more dangerous oxidizing product (Aitken *et al.*, 1993a). Hydroxyl radical is tremendously reactive and, hence, it can cause biological damage. Cellular homeostasis is normally regulated by the efficacy of the free-radical scavenger systems, by the concentrations of peroxidizable substances, such as PUFA, that are present in significant amounts and by an elevated concentration of docosahexaenoic acid (DHA) (C22:6 *omega-3*) fatty acids. In mature spermatozoa, the high concentration of unsaturated lipids is associated with a relative paucity of oxyradical scavenger enzymes. This relative deficiency is probably due to the virtual absence of cytoplasm in mature sperm cells (Poulos and White, 1973; Jones *et al.*, 1979; Bielski *et al.*, 1983; Ollero *et al.*, 2001).

Table 1. The most important classes of radical oxygen species.

Radical	*Notation*
Superoxide anion	$O_2^{\cdot -}$
Hydroxyl	$OH^{\cdot}$
Peroxyl	$ROO^{\cdot}$
Nitric oxide	NO
Peroxynitrite	$ONOO^-$
Nitroxyl anion	NO^-
Peroxynitrous acid	HOONO
Hydrogen peroxide	H_2O_2
Singlet oxygen	$^1O_2$
Hydrochlorous acid	HOCl

In physiological amounts, ROS are involved in the control of normal sperm function (De Lamirande and Gagnon, 1993a,b, 1995; Aitken and Fisher, 1994; Griveau *et al.*, 1994; Aitken, 1995; Griveau and Le Lannou, 1997). Paradoxically, spermatozoa necessitate a slight intracellular production of superoxide anion radical to boost the capacitation process (De Lamirande and Gagnon, 1993a,b) and the acrosome reaction (Griveau *et al.*, 1995a). The short half-life and limited diffusion of these molecules is consistent with their physiological role in maintaining the stability between ROS production and the scavenger systems. The balance between the amounts of ROS produced and the amounts scavenged at any moment determines whether a given sperm function will be promoted or compromised (Sharma and Agarwal, 1996). Recent data established that the upper cut-off value of normal semen samples that correlates with good semen quality is in the order of $0.075–0.1 \times 10^6$ counted photons/minute/10 million cells (Das *et al.*, 2008). In addition to the World Health Organization (1999) semen analysis, a study showed that patients with asthenozoospermia, asthenoteratozoospermia or oligoasthenoteratozoospermia have a significantly lower seminal plasma level of total antioxidant capacity (TAC) compared with a group of 16 healthy males with normozoospermia (Khosrowbeygi and Zarghami, 2007).

Two different pathways contribute to ROS production and the ensuing male subfertility or infertility: (i) the reduced NADPH oxidase system at the level of the sperm plasma membrane (Aitken *et al.*, 1992), which produces superoxide that is further converted to peroxide by the action of a SOD (Griveau and Le Lannou, 1997); and (ii) the reduced NAD-dependent oxido-reductase (diphorase) at the mitochondrial level (Gavella and Lipovac, 1992).

Very recently, the subcellular origin of sperm ROS has been further clarified. Disruption of mitochondrial electron transport flow in human spermatozoa results in the generation of ROS. The induction of ROS on the matrix side of the inner mitochondrial membrane at complex I causes a peroxidative damage of the midpiece and a loss of sperm movement. These findings suggest that sperm mitochondria

contribute to the oxidative stress of defective human spermatozoa (Koppers *et al.*, 2008).

Potential aetiological factors for oxidative stress in semen

There is much evidence that ROS are elevated in the male partners of infertile couples suffering from selected andrological diseases (D'Agata *et al.*, 1990; Mazzilli *et al.*, 1994). It has been postulated that ROS hyperproduction is a major cause of idiopathic male infertility and a reduced antioxidant capacity can contribute to this disease (Balercia *et al.*, 2003). Indeed, about 40% of infertile patients have a high ROS production, whereas only a minority of fertile men have increased seminal ROS production (Iwasaki and Gagnon, 1992). The most relevant pathologies (proposed as aetiological factors) that can increase ROS concentrations are described below.

Conditions in the scrotum

Scrotal temperature is increased by fevers, modifications in microcirculation, venous stasis such as in the presence of varicoceles. Ischaemia and hypoxia also increase ROS concentrations (Jung *et al.*, 2001; Paul *et al.*, 2009).

Infection/inflammation of the male organs

Infection/inflammation of the testis, epididymis, seminal vesicles and/or prostate may cause an increase in the number of seminal leukocytes (white blood cells; WBC) and/or WBC activation followed by an ROS burst, produced as a defence mechanism. This may be modulated via direct cell-to-cell contact or by soluble substances released by WBC (Saleh *et al.*, 2002a). Very recently, it has been shown that cytokines released during inflammation amplify the degree of OS initiated by WBC (Fraczek *et al.*, 2008). Alternatively, the antioxidant defence mechanisms can be overwhelmed resulting in OS (Sikka, 2001).

Oestrogen disorders

Oestrogens are either produced by an endogenous disequilibrium in androgen metabolism in the male reproductive tract or can access the reproductive tract via environmental exposure. Bennetts *et al.* (2008) reported that catechol oestrogens, quercetin, diethylstilbestrol and pyrocatechol were intensely active in stimulating redox activity, while genistein was only active at the highest doses tested and 17β-oestradiol, nonylphenol, bisphenol A and 2,3-dihydroxynaphthalene were inactive. It has been shown that ROS generation could be triggered by *cis*-unsaturated fatty acids including linoleic and DHA. This is of great importance because defective human spermatozoa contain abnormally high amounts of *cis*-unsaturated fatty acids, which may precipitate the OS encountered in male infertility (Aitken *et al.*, 2006). In this condition, ROS hyper-production damages sperm function, such as motility, capacitation, fertilization capability, acrosome reaction and DNA/chromatin integrity (Sikka, 1996; Aitken, 1997) (**Figure 1**).

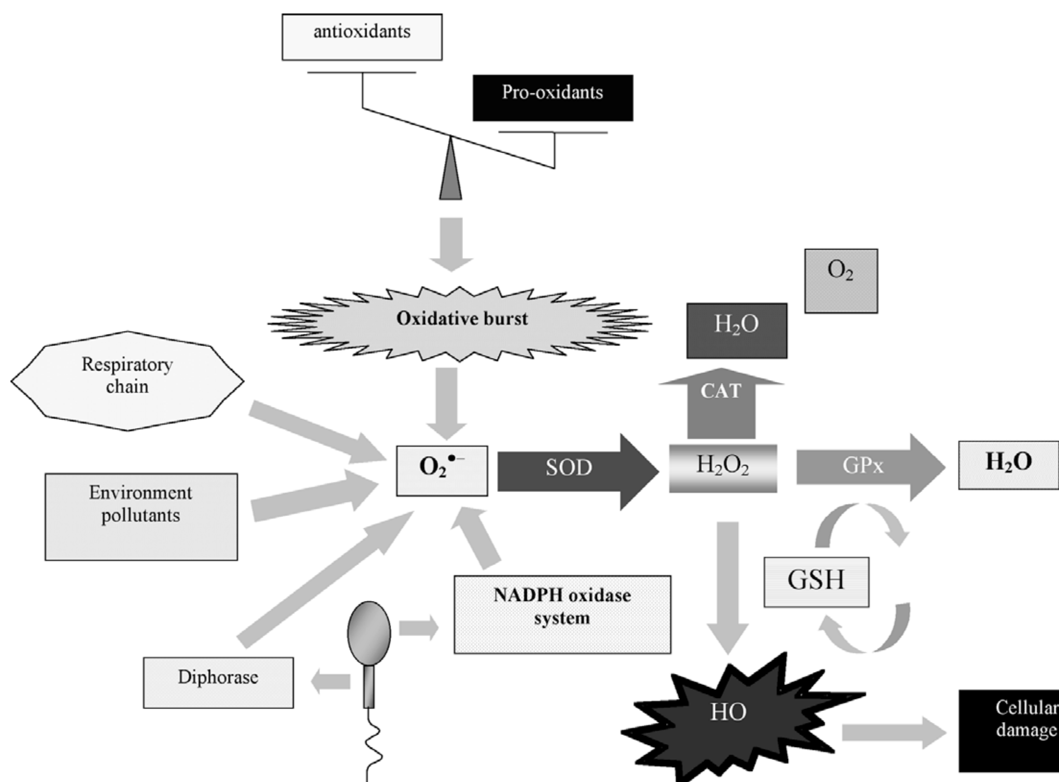

Figure 1. Schematic representation of reactive oxygen species-induced cellular damage. CAT = catalase; GPx = glutathione peroxidase; GSH = reduced glutathione; SOD = superoxide dismutase.

Chronic prostatitis

Men with chronic prostatitis, with or without leukocytospermia, have OS (Pasqualotto *et al.*, 2000). Production of ROS during the inflammatory processes of the testis and epididymitis are particularly harmful to spermatozoa. Indeed, a recent study, conducted in male Wistar rats, showed that repeated (1–2 weeks) experimentally induced exposure to the pro-oxidants *tert*-butyl hydroperoxide and cumene induced a marked dose-related enhancement of lipid peroxidation and increased ROS concentrations in both testis and epididymal spermatozoa (Kumar and Muralidhara, 2007).

Polymorphonuclear neutrophils

Polymorphonuclear neutrophils appear to be the major source of ROS (Tamura *et al.*, 1988; Ochsendorf, 1999; Aitken and Baker, 2006). Although these data look very worrying, only very high numbers of ROS-producing WBC in the final ejaculate are detrimental to sperm function. An infective/inflammatory injury involving ROS in the prostate gland, seminal vesicles or epididymis could indirectly impair sperm function (Ochsendorf, 1999).

Other factors

ROS can also be produced by normal and especially abnormal spermatozoa (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987; Fisher and Aitken, 1997; Aitken and Baker, 2006). Immature spermatozoa with abnormal head morphology and cytoplasmic retention produce the highest amount of ROS, whereas mature spermatozoa and immature germ cells produce the lowest amount. The oxidative damage of mature spermatozoa by immature sperm-produced ROS during sperm migration from the seminiferous tubules to the epididymis may be another cause of male infertility (Gomez *et al.*, 1996). Recently, serum and seminal plasma Cu concentrations have been found higher in subfertile men than in fertile men. Moreover, subfertile men have significantly higher seminal plasma Fe concentrations. These findings suggest that Cu and Fe might be mediators of the effects of oxidative damage and play an essential role in spermatogenesis and male infertility (Aydemir *et al.*, 2006). In addition, it has recently been shown that men older than 40 years have significantly higher ROS concentrations compared with younger men and a positive correlation between seminal ROS concentrations and age ($r = 0.20$; $P = 0.040$) has been reported (Cocuzza *et al.*, 2008).

Genetic dispositions in sperm oxidative stress

The discovery of specific genes and pathways affected by oxidants gives ROS a new function as second subcellular messengers in gene regulatory and signal transduction pathways (Allen and Tresini, 2000; O'Flaherty *et al.*, 2006) and specifically as physiological mediators that trigger phosphorylation events. The role of ROS as regulators of protein tyrosine phosphorylation has been known for a decade (Leclerc *et al.*, 1997), but other novel, ROS-mediated phosphorylations have been recently reported. These include phosphorylation of protein kinase A substrates and subsequently the phosphorylation of mitogen-activated kinase-like proteins, proteins with the threonine–glutamine–tyrosine motif and, finally, fibrous sheath proteins (O'Flaherty *et al.*, 2006; De Lamirande and O'Flaherty, 2008). A recently published article has offered a different point of view about OS, suggesting that sperm susceptibility to OS is significantly greater in idiopathic infertile men with the glutathione *S*-transferase mull 1 (GSTM1) null genotype compared with those possessing the gene. Therefore, the GSTM1 polymorphism might be an important source of variation in susceptibility of spermatozoa to oxidative damage in patients with idiopathic infertility (Aydemir *et al.*, 2007).

Sperm polyunsaturated fatty acid content

Spermatozoa are very susceptible to OS by virtue of their high content of PUFA as major components of cellular and intracellular membranes, the low cytoplasmic concentrations of scavenging enzymes and the small cytoplasmic volume, which limits their scavenging capacities and the lack of DNA repair capacity (Lenzi *et al.*, 1996). The reactivity of ROS, particularly hydrogen peroxide and the superoxide anion radical, has been proposed as a major cause of PUFA peroxidation in the sperm plasma membrane, playing a key role in the aetiology of male infertility (Sharma and Agarwal, 1996). The lipids of the spermatozoa have been suggested to be essential for their viability, maturity and function (Davis, 1981; Sebastian *et al.*, 1987). Phospholipids are the major structural components of membranes. Their fatty acid composition has been illustrated in a study by Zalata and colleagues (1998).

In normozoospermic samples, PUFA content ranges between 25.6% and 34% of total fatty acids and phospholipids in the 47% and 90% Percoll fractions, respectively. DHA contributes to more to than 60% of total PUFA; palmitate (C16:0) and stearate (C18:0), predominate among the saturated fatty acids of spermatozoa phospholipids. The omega-6/omega-3 ratio increases significantly in both Percoll fractions of samples with oligozoospermia or with asthenozoospermia compared with normozoospermic samples (Zalata *et al.*, 1998). Plasma membrane fluidity, conferred by PUFA, is crucial to regulate some specific functions, such as the acrosome reaction and the spermatozoa–oocyte fusion. PUFA and cholesterol are the main targets for lipoperoxidation. Their degree of unsaturation is, therefore, an essential parameter for the ability of spermatozoa to preserve equilibrium in an oxidative environment (Israelachvili *et al.*, 1980; Meizel and Turner, 1983; Alvarez and Storey, 1995; Ollero *et al.*, 2001). When ROS damage the double bonds associated with PUFA, a lipid peroxidation chain reaction begins. The most important outcome of this is a modification in membrane fluidity that can alter its function and consequently inhibits events during gamete fusion (Lenzi *et al.*, 1994).

The lower proportion of DHA, total PUFA, total omega-3 fatty acids, and the double-bond index in spermatozoa from both Percoll fractions of oligozoospermic patients and in the 90% Percoll fraction of asthenozoospermic samples could be the consequence of excessive breakdown of PUFA due to the increased ROS production in these samples (Aitken *et al.*, 1989; Zalata *et al.*, 1995, 1998). Most of the long-chain metabolites prejudice fertility in men with oligoasthenozoospermia, due to, at least in part, the reduced fluidity of the sperm membrane. Also, the significant increase of omega-6/omega-3 ratio in both oligozoospermic and asthenozoospermic, in comparison with normozoospermic samples, may suggest a physiological meaning of this ratio because of specific interactions of omega-6 and omega-3 fatty acids with certain membrane proteins and receptors (Lee *et al.*, 1986). The higher the number of PUFA double bonds, the greater is the peroxidative damage induced by ROS; for this reason, the human sperm plasma membrane, which is very rich in PUFA and contains those with two or more double bonds, especially docosapentanoic acid (that contains six double bonds), is very vulnerable to peroxidation (Kim and Parthasarathy, 1998).

In contrast to Zalata and colleagues (1998), Khosrowbeygi and Zarghami (2007) reported a significant difference in oleic acid concentrations in spermatozoa from asthenozoospermic men compared with normozoospermic men. In spermatozoa from asthenoteratozoospermic and oligoasthenoteratozoospermic men, all the tested fatty acids are significantly higher than those found in normozoospermic men. Seminal plasma catalase (CAT) concentrations were significantly lower in all patients, while concentrations of free 15-F(2t)-isoprostane were significantly higher in all patients compared with normozoospermic men. These results let us postulate that spermatozoa from abnormal samples may have higher concentrations of PUFA, especially DHA, than spermatozoa from normozoospermic men. Therefore, lipid peroxidation would be higher in spermatozoa from abnormal samples than those from normozoospermic men.

Oxidative stress damages sperm function

Several reports suggest that an increased production of ROS and/or modification in the levels of antioxidant defences are implicated in the occurrence of many sperm defects. These include reduction of sperm motility (De Lamirande and Gagnon, 1992a,b; Aitken *et al.*, 1993b; Sikka, 1996; Aitken, 1997), spermatozoa–oocyte fusion (Aitken *et al.*, 1991; Griveau and Le Lannou, 1997) and acrosine activity (Zalata *et al.*, 2004). It has been reported that more than half (55%) of the oligozoospermic patients who display a spermatozoa–oocyte penetration rate lower than 25% have an elevated ROS production (Aitken *et al.*, 1989). Furthermore, spermatozoa of oligozoospermic patients have been confirmed as a very important source of ROS (Aitken *et al.*, 1989; Zalata *et al.*, 1995). In addition, a strong correlation between sperm function, including motility, and the percentage of ROS-producing spermatozoa has been reported (Gil-Guzman *et al.*, 2001).

Some studies (Rao *et al.*, 1989; Kim and Parthasarathy, 1998) reported midpiece abnormalities, and some others showed that ROS-induced motility decrease is associated with a growth of lipid peroxidation measured as malondialdehyde (MDA) (Suleiman *et al.*, 1996; Chen *et al.*, 1997; Hsieh *et al.*, 2006) and DNA modifications (Chen *et al.*, 1997). Recently, it has been reported that the percentage of immotile spermatozoa correlate positively with MDA seminal plasma concentrations ($r = 0.50$, $P < 0.01$), while sperm concentration displays a significant negative correlation ($r = -0.63$, $P < 0.001$) (Saraniya *et al.*, 2008). On the contrary, a decrement of MDA corresponds to an increase of the pregnancy rate (Suleiman *et al.*, 1996) and an augmentation of ROS to fertility reduction *in vivo* (Aitken *et al.*, 1991). Very recently it has been shown that age affects the epidydimal antioxidant defence with an increased ROS production and consequent lipid peroxidation in Brown Norway rats (Weir and Robaire, 2007).

It has also been postulated that OS could be a cause for hyperviscosity of seminal plasma in infertile males (Aydemir *et al.*, 2008).

Oxidative stress and sperm chromatin and DNA integrity

Usually, sperm chromatin is condensed and insoluble; these features protect the genetic integrity and facilitate the transfer of the paternal genome through the male and female reproductive tracts. Furthermore, a special kind of protection against OS induced by metals is conferred to protamine by its capacity to trap some of them (Manicardi *et al.*, 1998; Liang *et al.*, 1999). Despite this tight DNA packaging and the seminal plasma protection from oxidative damage, many correlations have also been observed between ROS generation and DNA alteration (Lee *et al.*, 1986; Manicardi *et al.*, 1998; Twigg *et al.*, 1998a; Zalata *et al.*, 1998; Liang *et al.*, 1999). The exposure of spermatozoa to iatrogenically induced ROS significantly increases DNA fragmentation, modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements above that of the normal population (Aitken *et al.*, 1998; Barroso *et al.*, 2000). Indeed, ROS have been shown to induce: (i) DNA protein cross-linking in chromatin (Nackerdien *et al.*, 1991; Oliski *et al.*, 1992; Twigg *et al.*, 1998b); (ii) significant positive correlation with DNA fragmentation; (iii) high frequency of DNA single- and double-strand breaks (Liang *et al.*, 1999; Barroso *et al.*, 2000; Dizdaroglu, 1992; Chiu *et al.*, 1995); and (iv) oxidative DNA base changes, in a wide variety of mammalian cell types, especially in asthenozoospermic infertile and normozoospermic infertile subjects compared with fertile men (Hughes *et al.*, 1996; Kodama *et al.*, 1997). DNA fragmentation seems to be inversely correlated with sperm count, morphology, motility and fertilization rate (Sun *et al.*, 1997; Twigg *et al.*, 1998b; Shen and Ong, 2000; Aitken and Krausz, 2001). Several observations suggest that disorders in the DNA organization in the sperm nucleus are negatively related with the fertility competence of spermatozoa (Evenson *et al.*, 1999; Host *et al.*, 1999, 2000a,b; Shen *et al.*, 1999; Spanò *et al.*, 2000).

A higher percentage of DNA-damaged cells has been reported in the raw semen samples of patients with male accessory gland infection (Comhaire *et al.*, 1999). It was also described that chromatin alterations were higher in immature spermatozoa (Alvarez *et al.*, 2002). It is suspected that DNA damage may lead to an amplified risk of miscarriage and chromosomal abnormalities (Griveau and Le Lannou, 1997).

Within the fertilized oocyte, sperm DNA damage can be repaired during the period between sperm entry into the cytoplasm and the beginning of the next S phase, by virtue of pre- and post-replication mechanisms (Matsuda and Tobari, 1989; Genesca *et al.*, 1992; Evans *et al.*, 2004). Consequently, the biological impact of abnormal sperm chromatin structure depends on the combined effects of chromatin damage in the spermatozoa and the capability of the oocyte to repair that pre-existing damage. However, if spermatozoa are selected from samples with extensively damaged DNA for use in assisted reproduction treatment such as intracytoplasmic sperm injection (ICSI) or IVF, the oocyte's repair capacities might be inadequate, leading to fragmentation and a low rate of embryonic development that results in a high rate of early pregnancy loss (Ahmadi and Ng, 1999a,b) or a poor blastocyst development (Seli *et al.*, 2004). Sperm DNA fragmentation does not correlate with the fertilization rate, but there is a significantly reduced pregnancy rate in IVF patients inseminated with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling-positive spermatozoa. The same study showed a similar tendency in ICSI patients (Henkel *et al.*, 2003). This implies that spermatozoa with damaged DNA are able to fertilize oocytes, but at the time when the paternal genome is switched on, further development stops (Evenson *et al.*, 2002). Some reports have indicated that when >30% of spermatozoa have damaged DNA, natural pregnancy is not possible (Evenson *et al.*, 2002; Angelopoulou *et al.*, 2007; Evenson and Wixon, 2008). Other studies have, however, failed to confirm that this cut-off point affects treatment outcome (Payne *et al.*, 2005). It is noteworthy that the American Society for Reproductive Medicine recently reported that none of the current methods available to evaluate sperm DNA integrity predicts treatment outcome (Practice Committee of American Society for Reproductive Medicine, 2008). Therefore, in the presence of low OS, spermatozoa are still able to fertilize the oocytes, but at higher levels, DNA damage occurs. Repair of this kind of damage in the zygote can be anomalous and may lead to mutations linked with pre-term pregnancy loss and many pathologies in the offspring, including childhood cancer (Aitken and Baker, 2006).

Smoking, ROS production and sperm damage

Other causes may increase ROS production. Many studies have been carried out on the effect of cigarette smoking and show a decline in sperm count, motility, citric acid concentration and a rise in the number of abnormal cells (Sofikitis *et al.*, 1995; Saleh *et al.*, 2002b; Kunzle *et al.*, 2003). Men who smoke cigarettes present a 48% increase in WBC number, 107% higher ROS concentrations and a 10-point decrease in TAC scores with respect to infertile non-smokers. Using the sperm chromatin structure assay, it has been reported that the DNA fragmentation index is significantly higher in infertile men who smoke (Potts *et al.*, 1999). It has also been seen that cigarette smoke causes oxidative DNA damage in spermatozoa due to its high content of oxidants and its depletion of antioxidants.

Concentrations of Cd, Pb, MDA, protein carbonyls and ROS concentrations in infertile men who smoke have been reported to be significantly higher than those in fertile and non-smoking infertile men. Reduced glutathione (GSH) concentrations and glutathione *S*-transferase activity are lower in infertile smoker men than in fertile or non-smoking infertile men. Positive correlations have been found between seminal plasma Cd and seminal plasma protein carbonyls and between seminal plasma Pb and spermatozoon ROS concentrations in subfertile smokers, while there was a significant positive correlation between blood Cd and ROS concentrations in fertile smokers. A significant negative correlation between blood Cd concentrations and sperm and seminal plasma GSH concentrations have been reported (Kiziler *et al.*, 2007). Recently, cigarette smoking has been negatively correlated with a decrease of antioxidant activity, measured against SOD in the seminal plasma (Pasqualotto *et al.*, 2008). It has been shown that smokers have decreased seminal plasma vitamin E and vitamin C concentrations (Fraga *et al.*, 1996; Mostafa *et al.*, 2006).

In view of this, spermatozoa from smoking men exhibit augmented DNA damage. This may result in sperm DNA mutations that predispose offspring to greater hazard of malformations, cancer and genetic diseases (Ji *et al.*, 1997; Sépaniak *et al.*, 2006). Accordingly, epidemiological studies of childhood cancer have established that a paternal smoking habit is the most important identifiable risk factor linked with the beginning of the disease (Sorahan *et al.*, 1997a,b).

The sperm protective system against oxidative stress

Seminal plasma plays a crucial, protective role against ROS; its removal during sperm preparation may be hazardous to sperm DNA integrity (Jeulin *et al.*, 1989; Villegas *et al.*, 2003). The use of spermatozoa for ICSI will carry the same hazard by excluding the protective role of the seminal plasma (Liu *et al.*, 1994; Zorn *et al.*, 2003). These studies indicate that spermatozoa from patients with abnormal sperm count, motility and morphology have increased degrees of DNA damage. These data are in keeping with the similarly low value (~20%) reported by another study conducted using a group of unselected semen donors (Evenson *et al.*, 1991). Moreover, human seminal plasma appears to contain sufficient free Fe and Cu to catalyse the ROS-generating process (Kwenang *et al.*, 1987).

The fact that generation of ROS is liable to be elevated in severely oligozoospermic patients treated by ICSI only exacerbates the amount of oxidative damage which sperma-

tozoa undergo with this form of treatment (Aitken *et al.*, 1989; Zalata *et al.*, 1995). However, functionally competent spermatozoa are not a prerequisite for ICSI (Zorn *et al.*, 2003) and OS does not appear to interfere with fertilization rates achieved with this therapeutic technique (Twigg *et al.*, 1998b).

However, the ability of genetically damaged spermatozoa to achieve normal fertilization following ICSI could have adverse consequences. These may appear during the post-implantation development of the offspring, rather than before (Twigg *et al.*, 1998b). High seminal ROS concentrations are associated with impaired fertilizing ability and lower pregnancy rates after IVF. In ICSI, a negative association of ROS with embryo development to the blastocyst stage has been observed (Zorn *et al.*, 2003).

The vulnerability of ICSI arises from the fact that, with conventional IVF, the kind of OS that damages the genome also leads to collateral peroxidative damage to the sperm plasma membrane that may prevent spermatozoa–oocyte fusion from taking place. However, this fertilization block is removed when ICSI is performed. Thus, spermatozoa exhibiting severe DNA oxidative damage are able to produce normal rates of nuclear decondensation and pronucleus formation following ICSI (Twigg *et al.*, 1998b). It is likely that the oocyte and cleavage-stage embryo is competent to repair a certain degree of DNA damage (Genesca *et al.*, 1992). Evidence suggests the possibilities of the male infertility transmission to offspring (Bofinger *et al.*, 1999; Jiang *et al.*, 1999; Cram *et al.*, 2000; Aboulghar *et al.*, 2001).

Under physiological conditions, protection against OS is supplied to spermatozoa by numerous antioxidants, which are present both in the seminal plasma and spermatozoa. GSH, glutathione peroxidase (GPX), glutathione reductase (GRD) (Li, 1975; Alvarez and Storey, 1989), SOD (Alvarez *et al.*, 1987), glucose-6-phosphate dehydrogenase (Griveau *et al.*, 1995b), ascorbate (Lewis *et al.*, 1997), α-tocopherol (Therond *et al.*, 1996), taurine and hypotaurine (Holmes *et al.*, 1992) and coenzyme Q10 (CoQ10) (Lewin and Lavon, 1997) are the constituents of the antioxidant activity of the spermatozoon. An additional contribution is performed by lactoferrin that coats sperm heads, but it is secreted by seminal vesicles (Buckett *et al.*, 1997).

Antioxidant activity of the seminal plasma

Seminal plasma affords spermatozoa with a key defence against OS by several forms of ROS (Lenzi *et al.*, 1996; Balercia *et al.*, 2003), surrounding spermatozoa with a highly specialized scavenger system that preserves the cell membrane (Kim and Parthasarathy, 1998). Seminal plasma represents the most important defence against free-radical toxicity. It contains high and low molecular weight factors. They include enzymatic ROS scavengers, such as SOD (Nissen and Kreysel, 1983; Kobayashi *et al.*, 1991), which have been correlated with the liquefaction process of the seminal plasma and with the redox cycle of vitamin C, CAT (Jeulin *et al.*, 1989; Siciliano *et al.*, 2001) and non-enzymatic chain-breaking antioxidants such as vitamin C (Thiele *et al.*, 1995), vitamin E (Therond *et al.*, 1996), uric acid (Ronquist and Niklasson, 1984; Thiele *et al.*, 1995), albumin (Elzanaty *et al.*, 2007), carnitine, carotenoids and flavonoids (Tremellen *et al.*, 2007) and the amino acids taurine and hypothaurine (Holmes *et al.*, 1992), Zn (Gavella and Lipovac, 1998) and Cu (Nissen and Kreysel, 1983). A recent additional study has confirmed the protective role of SOD against lipid peroxidation (Tavilani *et al.*, 2008).

A special antioxidant attribute of the seminal plasma is the relatively high concentration of another non-enzymatic antioxidant: GSH. This is a tripeptide thiol constituting a cofactor of the selenium containing GPX, the main enzyme involved in converting H_2O_2 to alcohol and a substrate in reactions catalysed by glutathione transferase (an enzyme which catalyses covalent reactions of GSH with electrophilic substances such as quinones) (Aydemir *et al.*, 2007). In different biological systems, the glutathione redox cycle, involving the enzymes GPX and GRD, has an important role in protecting cells against oxidative damage (Reglinski *et al.*, 1988; Williams and Ford, 2004; Luberda, 2005; Tavilani *et al.*, 2008) through its thiolic group, which can react directly with hydrogen peroxide and the superoxide anion and hydroxyl radicals, and through its sulphydryl group, which can react with alkoxyl radicals and hydroperoxides, producing alcohols. Together, the enzyme scavengers and low-molecular weight antioxidants make up the TAC of the seminal plasma (Smith *et al.*, 1996).

One more defence is conferred by the prostasomes, secreted by the prostate into the seminal plasma. These organelles have the ability to interact with neutrophils and to reduce their capacity to produce superoxide anion radicals (Saez *et al.*, 1998). Prostasomes, in fact, can rigidify the plasma membrane of neutrophils and this results in the inhibition of the NADPH oxidase activity of neutrophils by lipid transfer from the prostasome to the plasma membrane of neutrophils (Saez *et al.*, 2000). Rhemrev *et al.* (2000) showed that the high antioxidant capacity of seminal plasma protects spermatozoa from OS, indicating also a different role of antioxidants contributing, respectively, to slow and fast total radical-trapping potential capacity. Seminal plasma has also been shown to be able to scavenge all of the considered oxyradicals with a similar efficiency for peroxyl and hydroxyl radicals, but with a slightly lower efficiency for peroxynitrite (Balercia *et al.*, 2003).

Antioxidant activity of spermatozoa

The existence of one alternative defence mechanism that safeguards spermatozoa from the action of ROS has been proven. The sperm heads have another kind of defence: lactoferrin. This is an iron-binding protein that coats the head avoiding the peroxidative action of the transition metal (Buckett *et al.*, 1997).

SOD and GPX/GRD pair play an important role against the deleterious effects of superoxide anion radical and hydrogen peroxide (Li, 1975; Nissen and Kreysel, 1983; Alvarez *et al.*, 1987; Alvarez and Storey, 1989) but also SOD, GPX and glucose-6-phosphate dehydrogenase

(Griveau *et al.*, 1995b) act together against the heart of hydrogen peroxide (Irvine, 1996). These enzymes seem to be present only in the cytoplasm of the sperm midpiece. Because of this localization, it seems unlikely that they can protect the sperm head plasma membrane and the tail.

The oxidation of ascorbic acid to dehydroascorbic acid produces the generation of both ascorbyl radicals and hydrogen peroxide. Since the concentration of CAT in spermatozoa and seminal plasma is low, GSH and GPX are the main agents that can eradicate the hydrogen peroxide generated (Luberda, 2005). The antioxidant system acts in an integrated fashion. SOD dismutates the superoxide anion radical into hydrogen peroxide. Produced hydrogen peroxide during the reactions has to be removed by the action of both CAT and GPX (Alvarez and Storey, 1989), GRD (Williams and Ford, 2004), α-tocopherol, vitamin C (Lewis *et al.*, 1997), vitamin E (Therond *et al.*, 1996), albumin (Elzanaty *et al.*, 2007) and taurine and hypotaurine (Holmes *et al.*, 1992). Generally, GSH is present in nanomolar concentrations in the cytosol, while its concentration is low in blood serum and in other biological fluids (Li, 1975).

Spermatozoa contain also the CoQ10, an energy-promoting agent with antioxidant properties, concentrated in the mitochondria of the midpiece. Its reduced form, ubiquinol, also acts as an antioxidant (Lewin and Lavon, 1997).

Epididymal antioxidant system

The epididymis also contains an enzymatic antioxidant system corresponding to GPX, SOD (Perry *et al.*, 1992, 1993) and CAT. In the mouse, a more specific type, such as indoleamine dioxygenase among these many kinds of GPX, plays an important role during the epididymal sperm transit (Drevet, 2006); SOD, GPX, glutathione transferase and the hexose monophospate shunt are present in the rat testis. These are variously expressed during the different stages of spermatogenesis (Yoganathan *et al.*, 1989; Peltola *et al.*, 1992). CAT is localized in peroxisomes, while GPX has been identified in the same subcellular organelles as SOD.

The role of vitamin E is to end the free-radical cascade in cellular membranes. Tocopheryl radicals are produced during the oxidation of vitamin E, which can then be reduced by ubiquinone or by ascorbic acid. The oxidation of vitamin C gives rise to ascorbyl radicals, which can be reduced by GSH and produce thiyl radicals and oxidized glutathione. This last step can then be reversed by GRD. Thus, the whole system has to work simultaneously, and an alteration of one of the components can lead to a potentially damaging accumulation of free radicals.

In spermatozoa of patients with oligozoospermia, GSH concentrations are significantly lower than in controls. Sperm GSH content in normozoospermic men shows a large variation. A significant association between the intracellular GSH content and the aptitude to penetrate bovine cervical mucus has been reported. The intracellular GSH concentrations correlate significantly with the GSH concentrations in seminal plasma. The GSH concentration in seminal plasma does not differ between the various groups, however, it correlates significantly with FSH serum concentrations (Ochsendorf *et al.*, 1998). Analogous findings have been reported by Lewis and colleagues (1997), who described decreased concentrations of ascorbate in the seminal plasma of asthenozoospermic men and improved ROS activity. Higher ROS production was observed in 16 of the 18 patients (88.8%, $P < 0.0001$ versus controls). Seminal plasma SOD, CAT, GPX and total sulphydryl-group concentrations in infertile patients were significantly lower than in controls (Alkan *et al.*, 1997; Pasqualotto *et al.*, 2008). It has been shown, that seminal plasma, TAC is generally lower in men with varicocele than in healthy subjects (Barbieri *et al.*, 1999; Hendin *et al.*, 1999).

While SOD may play a physiological role in maintaining a balance between superoxide anion radical and hydrogen peroxide, high concentrations of this enzyme are linked with impaired sperm function because: (i) there is excessive generation of hydrogen peroxide, which causes peroxidative damage; (ii) it impairs the fertilizing potential of the spermatozoa by removing superoxide anion radical; and (iii) high SOD activities reflect errors during the spermatogenesis associated with germ cell exfoliation and the retention of excess residual cytoplasm by the spermatozoa (Aitken *et al.*, 1996). When the scavenging capacity of the seminal plasma was related to sperm motility parameters, a significant relationship was found with total oxyradical scavenging capacity values towards hydroxyl radicals, demonstrating a lower protection against toxicity of these specific ROS in seminal plasma of individuals with reduced motility of sperm cells (Balercia *et al.*, 2003). However, the literature reports contradictory evidence of the occurrence of oxidative damage to human spermatozoa and modification of single antioxidant defence. The latter have been reported to increase, decrease or even remain constant in seminal plasma and spermatozoa of individuals influenced by a range of infertility problems (Alleva *et al.*, 1997; Lewis *et al.*, 1997; Ochsendorf *et al.*, 1998; Zini *et al.*, 2000a). In this respect, it should be considered that OS embraces a complex set of phenomena; thus, it is highly doubtful that the analysis of a single antioxidant can elucidate specific relationships between a mixture of stressors and cellular damage (Wayner *et al.*, 1987). An enhancement of oxidative damage to sperm membranes, proteins and DNA is linked with modification in signal transduction mechanisms that involve fertility (Sikka *et al.*, 1995). Despite the above-reported contrasting results, an effort to measure the antioxidant defences has been made. The concentrations of SOD and MDA both in the seminal plasma and spermatozoa were similar. With regard to GPX, it is about 13 times higher in spermatozoa than in the seminal plasma. Nitric oxide is also slightly higher in spermatozoa when compared with the seminal plasma (Gallardo, 2007).

In-vitro antioxidant supplements in human sperm preparation techniques

It has been confirmed that the techniques utilized for preparing spermatozoa have an effect on ROS production in human sperm suspensions and this inversely correlates

with the fertilizing potential of spermatozoa *in vitro* (Aitken and Clarkson, 1988). Therefore, it has been shown that IVF success rate is significantly improved when ROS production declines (Sukcharoen *et al.*, 1996). Adding medium before liquefaction may prevent the binding of bacteria and detritus to the sperm surface and subsequently decrease the DNA damage triggered by ROS (Zollner *et al.*, 2001).

Some techniques, such as swim-up from semen or density-gradient protocols (Percoll or PureSperm), have been established to significantly improve motility and morphology (Aitken and West, 1990) and to reduce the proportion of spermatozoa with DNA fragmentation (Colleu *et al.*, 1996; Sakkas *et al.*, 2000) and, consequently, to be a valid aid for semen preparation (Benchaib *et al.*, 2007). When density-gradient centrifugation and swim-up were compared, the results showed the latter approach to result in a better rate of curvilinear and straight-line velocity, hyperactivation, acrosome reaction (Poulos and White, 1973) and DNA integrity (Zini *et al.*, 2000b). In contrast, repeated centrifugation of washed sperm preparations and the isolation of spermatozoa from seminal plasma have been shown to increase ROS production and to damage sperm DNA, possibly due to the mechanical activation of cell membrane oxidative systems in addition to contact with damaged spermatozoa and WBC.

Although some studies on bovine oocytes (Blondin *et al.*, 1997) and embryos (Dalvit *et al.*, 1998) reported contradictory results, many data showed that supplementation of culture media with antioxidants can improve sperm quality and reduce OS in some animal species. Studies have been conducted on ram spermatozoa with different compounds at various concentrations showing improved sperm functions and pregnancy rates (Maxwell and Stojanov, 1996; Mara *et al.*, 2005). In bovines, disulphide-reducing agents or divalent cation chelators prolong the motility of spermatozoa after freezing–thawing (Lindemann *et al.*, 1988), vitamins E and C alone or in combination play a relevant role in improving oocyte fertilization (Blesbois *et al.*, 1993) and CAT reverses the reduction of the oocyte penetration rate induced by ROS (Blondin *et al.*, 1997). In rat spermatids, GSH can avoid the damage resulting from exposure to peroxidizing agents (Den Boer *et al.*, 1990).

The presence of antioxidants can suppress the generation of ROS (Aitken and Clarkson, 1988; Donnelly *et al.*, 1999) and antioxidants may protect sperm DNA. When added *in vitro*, vitamin C (600 mmol/l), α-tocopherol (30 and 60 mmol/l) and urate (400 mmol/l) each have been described to give significant protection ($P < 0.001$) from subsequent DNA damage by X-irradiation. Thus, the supplementation of the culture medium with antioxidant compounds separately can beneficially affect sperm DNA integrity (Hughes *et al.*, 1998). Some antioxidants such as ascorbate and α-tocopherol are able to provide significant protection against DNA damage (Donnelly *et al.*, 1999) and exhibit anti-apoptotic effects in a variety of cell culture systems, including granulosa cells and antral follicles (Kolodecik *et al.*, 1998; Tarín *et al.*, 1998).

Vitamin C is a water-soluble ROS scavenger with high potency. It is capable of downgrading peroxidation outside the cell but has little effect in the membrane or inside the cell. Vitamin C has two different actions: at concentrations below 1000 μmol/l it protects spermatozoa from free-radical damage as shown by improvement in their motility and viability. Concomitantly, there is also depletion of malondialdehyde generation (an end product of lipoperoxidase). At a concentration higher than 1000 μmol/l vitamin C is, however, a pro-oxidant, as shown by an abrupt fall in sperm motility and viability and concomitant increase in lipid peroxidation (Verma and Kanwar, 1998).

Effectiveness of antioxidants is often linked to the cause of the ROS production. Parinaud and colleagues (1997) have shown that the supplementation of antioxidants (Sperm-Fit) during sperm centrifugation significantly reduces WBC-mediated motility loss. Moreover, the supplementation of albumin in the culture medium has been shown to protect spermatozoa from the detrimental action of ROS, mainly when ROS come from spermatozoa (Storey, 1997) and to increase the recovery of higher quality spermatozoa compared with Percoll (Armstrong *et al.*, 1998). *In vitro* studies on spermatozoa have established that supplementation of culture media with antioxidants counteracts asthenozoospermia (Parinaud *et al.*, 1997). Addition of GSH and hypotaurine, either singly or in combination, to sperm preparation medium had no significant effect on sperm progressive motility or baseline DNA integrity (Donnelly *et al.*, 2000).

Spermatozoa of patients with asthenozoospermia incubated with 50 μmol/l of CoQ10 show a significant increase in motility, while no effect is reported in spermatozoa with normal motility (Lewin and Lavon, 1997). Data on ferulic acid suggest that it is beneficial to sperm viability and motility in both fertile and infertile individuals, leading to a decline of lipid peroxidative damage to sperm membranes and increase of intracellular cAMP and cGMP (Zheng and Zhang, 1997). Ferulic acid, a *trans*-cinnamic acid derivative, is an organic compound of plant cell walls. As a component of lignin, ferulic acid is a precursor in the manufacture of other aromatic compounds. With dihydroferulic acid, it is a lignocellulose component, serving to cross-link the lignin and polysaccharides, thereby conferring rigidity to the cell walls. Ferulic acid can also be found in plants seeds such as rice, wheat and oats, as well as in coffee, apple, artichoke, peanut, orange and pineapple.

The use of GSH and SOD *in vitro* has also been proposed for improving sperm function. SOD increases hyperactivation and acrosome reaction rates, while GSH has been effective in improving acrosome reaction. CAT did not show any significant effect on these parameters (Griveau and Le Lannou, 1994). These results denote that GSH safeguards sperm motility *in vitro* during pelleting, when they come into contact with seminal ROS, produced by WBC or damaged spermatozoa (Lenzi *et al.*, 1998).

Sperm SOD activity confirms a significant correlation with the number of motile spermatozoa, whereas seminal plasma SOD activity does not relate to sperm concentration or

motility. MDA sperm concentration is significantly associated with the number of immotile spermatozoa. A decline in the motility of spermatozoa incubated in medium devoid of seminal plasma is observed after 120 min while the MDA concentration of the spermatozoa increased. Supplementation of exogenous SOD (400 U/ml) to the sperm suspension significantly reduced this loss of motility and the augmentation of the MDA concentration. These findings propose a significant role for SOD in sperm motility. It seems that lipid peroxidation of human spermatozoa may cause loss of motility and that SOD may avoid this lipid peroxidation. These results suggest that SOD may have a possible clinical application in the use of spermatozoa prepared for assisted reproduction treatment (Kobayashi *et al.*, 1991).

Isoflavones (genistein and equol) are plant compounds. Their physiological effects include antioxidant activity. Compared with vitamin C and α-tocopherol, genistein was the most potent antioxidant, followed by equol. Genistein and equol, when added in combination, were more protective than when added singularly. Based on these preliminary data, these compounds may play a role in antioxidant protection against sperm DNA damage (Sierens *et al.*, 2002).

Pentoxifylline has a stimulating effect on Fe-induced lipid peroxidation, which usually acts positively on membrane fluidity and physiological destabilization. However, it can also stimulate a damaging peroxidation chain reaction when the spermatozoa are weaker than usual or when incubation is too long (Gavella and Lipovac, 1994). Spermatozoa from 15 asthenozoospermic patients whose spermatozoa formed high concentrations of ROS at steady state were treated *in vitro* with pentoxifylline to verify its effect on ROS production and sperm motion parameters. Pentoxifylline diminished ROS generation by spermatozoa in these patients and preserved the decrease of curvilinear velocity and beat cross-frequency for 6 h *in vitro* (Okada *et al.*, 1997).

In a recent study, spermatozoa washed with Ham's F-10 media, incubated with EDTA and various CAT concentrations generated a significantly lower amount of ROS compared with spermatozoa incubated without these compounds. CAT significantly increased sperm acrosome reaction rate. Both the antioxidants significantly reduced the DNA fragmentation rate of the spermatozoa, whereas no effect on lipid peroxidation was observed (Chi *et al.*, 2008). Another recent study on Boer bucks spermatozoa has shown that motility is improved and DNA damage is reduced after incubation with α-lipoic acid at a concentration of 0.02 mmol/ml (Ibrahim *et al.*, 2008).

Antioxidant therapy in human male infertility

Despite contrasting results (Ten *et al.*, 1997; Ménézo *et al.*, 2007), antioxidant therapy appears to be efficient not only *in vitro* but also *in vivo* as an efficient strategy to improve the reproductive function. Experimental data in laboratory and farm animals support this contention (Chew, 1993; Luck *et al.*, 1995). After exposure to ROS, the sperm membrane becomes more fragile and antioxidant treatment may prevent lipid peroxidation of sperm membranes (Lenzi *et al.*, 1998). GSH therapy has a crucial role in increasing sperm motility of spermatozoa and consequently in improving fertilization in bulls with asthenozoospermia due to varicocele and in rabbits with dyspermy caused by cryptorchidism (Tripodi *et al.*, 2003). In lead-injected mice, the administration of vitamin C, at a concentration equivalent to the human therapeutic dose (10 mg/kg body weight), is able to significantly reduce the testicular MDA content with a simultaneous rise in sperm count and a significant reduction in the proportion of abnormal sperm population. Vitamin E (100 mg/kg body weight) treatment has a similar but lower efficacy than vitamin C. The co-administration of both vitamins at the above-mentioned doses leads to the most significant drop in MDA content along with elevation of sperm count and a decrease in the percentage of abnormal spermatozoa (Mishra and Acharya, 2004). Vitamin E treatment has a similar effect against mercury-induced alteration of sperm number and functions (Rao and Sharma, 2001). A high amount of dietary α-tocopheryl acetate significantly increases vitamin E semen concentrations and its oxidative stability after cryopreservation. When the seminal plasma ascorbate concentration decreases to 7.3 μg/ml, the fertilization rate and the hatching rate of embryos decreases significantly. When associated with higher vitamin E concentrations, ascorbate increased seminal plasma α-tocopherol concentrations and the oxidative stability of semen, while both parameters decrease with lower vitamin E concentrations. Their combination significantly improves the viability and the kinetics of spermatozoa with an increase in fertility rate (Castellini *et al.*, 2000).

The trials conducted on animal models indicate that antioxidant therapies can be successful in humans. Some of these studies suggest that a first-line therapy is prevention and this must be assured by an adequate dietary intake. Controversial data often result from the many uncontrolled studies carried out to support such a treatment and its efficacy is not yet proven (Agarwal and Said, 2004). Unfortunately, andrologists often see patients with fertility problems many years after the beginning of their pathology mainly because andrological diseases have subclinical effects and few or no symptoms. In this manner the sperm damage becomes often irreversible.

In vivo trials in humans have shown that administration of antioxidants improves sperm quality in heavy smokers (Dawson *et al.*, 1992) and in patients with male factor infertility (Lenzi *et al.*, 1993) as well as increasing the fertilizing potential of healthy men with high seminal ROS concentrations (Kessopoulou *et al.*, 1995) and fertile normozoospermic men with low fertilization rates in previous IVF cycles (Geva *et al.*, 1996).

Whether antioxidant therapy in men can be improved is an unsolved question, as high doses of certain antioxidants, including vitamin A, may have embryotoxic and teratogenic effects (Geelen, 1979; Tzimas and Nau, 2001). This review cannot exclude the possibility that selected patients with elevated ROS generation or with reduced protective

scavenging capacity in the seminal plasma may benefit from antioxidant treatment as suggested by Kessopoulou and Lenzi and their colleagues (Lenzi *et al.*, 1993; Kessopoulou *et al.*, 1995). But as yet, it is unknown whether ROS production can be used as a criterion to select men for antioxidative therapy, since intracellular sperm antioxidant status, sperm count, abstinence time and other confounding factors must also be considered. Also, no reliable, predictive and low-priced tests are available to evaluate the ROS exposure or to measure the TAC of the patient. Therefore, a valid approach would be to remove all causes that can amplify ROS production and/or to decrease seminal plasma scavenging activity.

To identify which markers can be useful to measure OS before starting any antioxidant treatment or, alternatively, which markers can better measure ROS-induced damage in the plasma membrane would be very useful. A method to quantify the OS-induced damage will allow us to better evaluate the post-treatment efficacy and to understand how the injury will benefit from the antioxidant treatment. The main antioxidant compounds used in humans and their effect on the reproductive function are reviewed below.

Ascorbic acid (vitamin C)

In seminal plasma, vitamin C concentrations are 10-fold higher than in serum (Jacob *et al.*, 1992). Vitamin C is a powerful antioxidant when peroxyl radicals are present in the aqueous phase (Frei *et al.*, 1989), but the vitamin is a weak scavenger for ROS within the lipid membrane (Doba *et al.*, 1985). In semen samples with ROS hyper-production, ascorbate concentrations in seminal plasma are significantly reduced (Lewis *et al.*, 1997). Seminal ascorbic acid concentration is also significantly lower in leukospermic samples. A significantly greater percentage of samples with abnormal DNA fragmentation index has been detected in samples with low seminal ascorbic acid concentrations compared with those with normal or high concentration of ascorbic acid (Song *et al.*, 2006). Interestingly, at low concentrations, vitamin C is an antioxidant, but at high concentrations it can start an auto-oxidation process (Wayner *et al.*, 1986). In addition, plasma saturation of vitamin C takes place in humans at a daily amount of 1 g and higher doses may stimulate the formation of kidney stones because of the increased excretion as oxalate (Levine *et al.*, 1996).

A 2.2-fold increase in plasma ascorbic acid concentration is achieved with a supplementation dose of vitamin C (1 g/day) (Wen *et al.*, 1997). Furthermore, its seminal plasma concentrations correlated positively with the percentages of morphologically normal spermatozoa (Thiele *et al.*, 1995) and this evidence can also indicate that vitamin C is a protective vitamin in the epididymis.

In previous studies, attempts have been made to improve semen parameters of infertile men by vitamin C supplementation (1 g/day) (Dawson *et al.*, 1987; Dawson *et al.*, 1992). An elevated intake of vitamin C was related with improved semen quality, as indicated in the higher mean sperm count, sperm concentration and total progressive motile sperm count (Eskenazi *et al.*, 2005). In a placebo-controlled study in smokers, the groups receiving vitamin C at a dose of 200 or 1000 mg/day, had sperm parameter improvement, and the most relevant improvement was observed in the group receiving the highest dose for 4 weeks (Dawson *et al.*, 1992). Vitamin C protects human spermatozoa from endogenous oxidative DNA damage (Fraga *et al.*, 1991).

α-Tocopherol (vitamin E)

A single-blind study has been carried out with vitamin E. Eight patients receiving vitamin E at the dose of 100 mg three times a day for 120 days failed to show any improvement (Giovenco *et al.*, 1987). Administration of 300 mg/day of vitamin E determines a small rise in seminal plasma vitamin E concentration (Moilanen *et al.*, 1993). Its seminal plasma concentrations become faintly more elevated in infertile men when vitamin E is given at doses of 300 and 1200 mg/day for 3 weeks (Moilanen and Hovatta, 1995). The concentration of α-tocopherol in spermatozoa is independent from the concentration and the total α-tocopherol amount in the seminal plasma; the percentage of motile spermatozoa is significantly related to sperm α-tocopherol content (Therond *et al.*, 1996).

Efforts have been made to improve semen parameters of infertile men by vitamin E (600 mg/day) administration (Kessopoulou *et al.*, 1995). In a double-blind, randomized, placebo crossover controlled trial, 30 healthy men with high semen ROS concentrations and a normal female partner received vitamin E (600 mg/day) or placebo tablets for 3 months. Vitamin E increased significantly blood serum vitamin E concentrations and improved the *in vitro* sperm function as assessed by the zona-binding test (Kessopoulou *et al.*, 1995). Other studies used lower doses of vitamin E, such as 300 mg/day (Giovenco *et al.*, 1987; Moilanen *et al.*, 1993) or 200 mg/day (Geva *et al.*, 1996). A placebo controlled double-blind study showed that sperm MDA concentration was higher in asthenozoospermic and oligoasthenozoospermic patients and that vitamin E administration significantly reduced MDA concentration and enhanced sperm motility in asthenozoospermic men (Suleiman *et al.*, 1996). Furthermore, 11 (21%) of 52 spouses of the treatment group became pregnant in the course of the 6-month treatment period; resulting in nine normal-term deliveries, whereas the other two aborted in the first trimester. No pregnancies were reported in the placebo group (Suleiman *et al.*, 1996).

In a prospective study, 15 fertile normozoospermic men, who had low fertilization rates in their earlier IVF cycles, were treated with 200 mg/day of vitamin E for 3 months. The high MDA concentrations significantly declined to normal and the fertilization rate per cycle improved significantly after 1 month of treatment (Geva *et al.*, 1996).

In another study, 97 healthy, non-smoking men were interviewed on dietary habits and their semen was analysed. A high intake of daily nutrients and supplements with antioxidant quality was associated with a better semen quality; for example, vitamin E intake and progressive motility and total progressive motile sperm count; and between β-carotene intake and sperm concentration and progressive motility (Eskenazi *et al.*, 2005).

Ascorbic acid (vitamin C) and α-tocopherol (vitamin E)

Vitamin C and vitamin E may operate synergistically *in vivo* to reduce the peroxidative damage on spermatozoa, by joining their hydrophilicity and lipophilicity. In addition, if these agents act directly on spermatozoa to avoid damage by ROS, such improvement may be fast, provided that the vitamins gain access to spermatozoa either at ejaculation or within the epididymis. In patients with asthenozoospermia, a prominent production of seminal plasma ROS and a higher ROS-mediated injury of sperm membranes has been discovered, but the source of these effects is unidentified (De Lamirande and Gagnon, 1992a,b; Agarwal *et al.*, 1994). Neither is it known at which point the peroxidative damage to spermatozoa takes place, whether within semen (during the time required for liquefaction), during the epididymal transit or within the testis. By altering membrane integrity, ROS may prejudice sperm motility as well as sperm viability (Davis, 1981; Sebastian *et al.*, 1987).

In a single-centre, double-blind, placebo-controlled randomized study, simultaneous daily administration of high vitamin C (1 g) and vitamin E (800 mg) doses for 8 weeks did not improve semen parameters or 24-h sperm survival rate in patients with asthenozoospermia or moderate oligoasthenozoospermia (Rolf *et al.*, 1999). These disappointing results agree with those reported by some (Giovenco *et al.*, 1987; Moilanen *et al.*, 1993) but are at variance with those reported elsewhere in the literature (De Lamirande and Gagnon, 1992a; Geva *et al.*, 1996). It is possible that the relatively short treatment time utilized in this study explains why no improvement was found, especially if the effect takes place within the testis.

In another study, 64 men with unexplained infertility and an elevated percentage (≥15%) of DNA-fragmented spermatozoa in the ejaculate were randomly divided into two groups. One group received vitamin C (1 g) and vitamin E (1 g) daily and the other placebo. After 2 months of treatment, the percentage of DNA-fragmented spermatozoa was significantly reduced in the antioxidant-treated group, whereas no difference was observed in the placebo group (Greco *et al.*, 2005a). Another study was conducted on 38 men with an elevated proportion (≥15%) of DNA-fragmented spermatozoa in the ejaculate. They were treated with vitamin C (1 g) and vitamin E (1 g) daily for 2 months after one ICSI cycle failure. In 29 of these cases (76%), the antioxidant treatment led to a reduction in the percentage of DNA-fragmented spermatozoa and a second ICSI effort produced a large improvement in the clinical pregnancy (48.2% versus 6.9%) and implantation (19.6% versus 2.2%) rates (Greco *et al.*, 2005b).

α-Tocopherol (vitamin E) and selenium

There is only one study that attempted to treat, in an open randomized trial, 28 men with a daily administration of vitamin E (400 mg) and selenium (225 µg) for 3 months. In this study, another 26 patients received vitamin B (4.5 g/day) for the same duration. In these patients, vitamin E and selenium supplementation produced a significant decrease in MDA concentrations and an improvement of sperm motility (Keskes-Ammar *et al.*, 2003).

Glutathione

GSH seems to be the most frequently used compound, owing to its demonstrated antitoxic and antioxidant action in other degenerative pathologies. Although it cannot cross cell membranes, the concentration this antioxidant in biological fluids can increase after systemic administration. GSH is able to reach the seminal plasma and concentrate there. In this fluid, it protects spermatozoa from oxidative stress, suggesting that its supplementation may play a therapeutic role in some andrological disease, particularly during inflammation (Lenzi *et al.*, 1993).

In a 2-month pilot study, GSH (600 mg/day i.m.) was administrated to a group of patients with dyspermia associated with various selected andrological pathologies. A significant discrepancy was seen in the proportion of spermatozoa with forward motility and in the parameters of the sperm motility evaluated by computer analysis. Sperm motility increased, particularly in patients with chronic inflammation of the genital tract and in patients with varicocele (Lenzi *et al.*, 1992), two conditions in which ROS or other toxic compound production may play a pathogenic function. After these promising results, the same authors conducted a placebo-controlled double-blind cross-over trial on a group of infertile patients suffering from unilateral varicocele and germ-free genital tract inflammation. The patients were assigned to treatment with GSH 600 mg i.m. every other day or placebo preparation. All the selected patients showed an increase in sperm concentration and a highly statistically significant improvement in sperm motility, sperm kinetic parameters and sperm morphology. In the presence of these results, the authors suggested that the effect must be due to a post-spermatocyte action, as the treatment period was expressly chosen to be shorter than a complete spermatogenetic cycle and because early positive results were found after the first month of therapy. These effects on sperm motility and morphology lasted beyond the period of treatment. GSH acts indirectly by improving the metabolic condition of the testicular-epididymal environment (Lenzi *et al.*, 1993). These sperm alterations can be partially reversed by GSH therapy if the structural cell membrane damage is not too severe (Lenzi *et al.*, 1994).

These studies suggest that, at least in part, the therapeutic action of GSH is due to the biochemical modifications in membrane constitution and its resulting protective effect on the lipid components of the cell membrane. The reduction in the concentration of lipoperoxide in seminal plasma implies that GSH reduces the effect of the lipoperoxidative process produced by vascular or inflammatory pathologies.

Carnitines

Carnitines are implicated in many metabolic processes that are carried out by a number of cellular organelles. They play a fundamental role in the maturation of spermatozoa

within the male reproductive tract and have a crucial role in sperm metabolism by providing readily available energy for use by spermatozoa, which positively reflects on sperm motility and concentration (Tang *et al.*, 2008). The achievement and preservation of progressive motility take place in parallel to L-carnitine increase and accumulation in the epididymal lumen (Jeulin *et al.*, 1988).

A number of controlled and uncontrolled human and animal studies have been conducted to point towards a possible use of carnitines as antioxidants. As a result, treatment with carnitines may represent an option within a broader therapeutic approach in men with ROS-mediated infertility (Dokmeci, 2005). Some interesting results indicate that, in patients with prostato-vesiculo-epididymitis (PVE), antimicrobials and/or anti-inflammatory drugs get a full positive antimicrobial response but a partial antioxidative response, which seems to be potentiated by the addition of carnitines as third-line treatment. Furthermore, it is important to underline that the antioxidative treatment with carnitines administered simultaneously with anti-infectious agents is less effective. Finally this treatment is unsuccessful without the eradication of the pro-oxidant factors (germs and WBC) (Vicari *et al.*, 2001). In an open, prospective, randomized study, 98 patients with PVE and leukocytospermia, antioxidant treatment with carnitines was fully effective if these patients were pretreated with non-steroidal anti-inflammatory compounds (Vicari *et al.*, 2002).

A variety of studies sustain that L-carnitine and/or L-acetylcarnitine, at a total daily dose of at least 3 g, can significantly increase both sperm concentration and total sperm counts in patients with asthenozoospermia or oligoasthenozoospermia. Even if many clinical trials have shown the valuable property of carnitines in selected cases of male infertility, the majority of these trials have been conducted without placebo-control and double-blind design, making it complex to get a definite conclusion. Therefore, well-designed studies are needed to further validate the use of carnitines in the therapy of male infertility (Agarwal and Said, 2004). In a placebo-controlled, double-blind, crossover trial, L-carnitine was successful in improving semen quality, but it failed to decrease the lipid peroxidation potential. These results suggest a partial role of this compound in neutralizing ROS action (Lenzi *et al.*, 2003). In an another study, L-carnitine (2 g/day) and L-acetyl-carnitine (1 g/day) for 3 months in patients with PVE and elevated ROS production, showed that carnitines are an effective treatment only when seminal WBC are normal (Vicari and Calogero, 2001).

Coenzyme Q10

CoQ10 is a lipid-soluble element of the respiratory chain. It acts in its reduced form (ubiquinol), as a strong antioxidant in several biological systems, such as lipoproteins and membranes.

To assess the effect of CoQ10 administration *in vivo*, 17 patients with low fertilization rates after ICSI for male factor infertility were treated with oral CoQ10 (60 mg/day) for a mean of 103 days before the next ICSI treatment. A considerable improvement was achieved in terms of fertilization rate. In conclusion, the administration of CoQ10 may result in improvement in sperm functions in selective patients (Lewin and Lavon, 1997).

The content of CoQ10 in both its reduced and oxidized forms (ubiquinol/ubiquinone) and the hydroperoxide concentrations in seminal plasma and seminal fluid have been measured in 32 infertile patients. A significant correlation between ubiquinol content and sperm count was seen, as well as an inverse correlation between ubiquinol content and hydroperoxide concentrations. A significant correlation between sperm count, motility and ubiquinol-10 content in seminal fluid has also been established. An inverse correlation between ubiquinol/ubiquinone ratio and percentage of abnormal morphology was also observed in total fluid (Balercia *et al.*, 2004). These results suggest that ubiquinol-10 inhibits hydroperoxide formation in seminal fluid and in seminal plasma (Alleva *et al.*, 1997).

CoQ10 is present at remarkable concentrations in the human seminal fluid, and exhibits a direct correlation with sperm count and motility. In patients with varicocele, on the contrary, correlation with lack of sperm motility and a higher proportion of CoQ10 has been found in the seminal plasma (Mancini *et al.*, 1998). In this study, higher CoQ10 concentrations were found in spermatozoa of oligozoospermic and asthenozoospermic patients without varicocele. This relationship was not noted in patients with varicocele, who also showed slightly lower intracellular absolute values of the coenzyme. Higher intracellular concentrations may relate to a defence mechanism of the spermatozoa. In varicocele patients, this mechanism could be scarce, leading to higher sensitivity to oxidative damage (Mancini *et al.*, 1998).

Lycopene

Lycopene is a constituent of the human redox protection mechanism against free radicals. Oral lycopene treatment seems to play a role in the management of idiopathic male infertility. Significant improvement seems to take place in the sperm concentration and motility following the administration of 2 g of lycopene, twice a day for 3 months. Sperm concentration improvement begins, however, from a sperm density $>5 \times 10^6$/ml (Gupta and Kumar, 2002).

Pycnogenol

Pycnogenol is extracted from the bark of *Pinus maritima*. The biological precursors of the oligomeric procyanidins, such as catechin and taxifolin, are effective and well-known free-radical scavengers. Pycnogenol's components inhibit cyclo-oxygenases that produce inflammatory prostaglandins (Baumann *et al.*, 1980). Mean sperm morphology following Ham's F-10 capacitation increases by 38% following 3 months treatment with 200 mg/day pycnogenol, and the mannose receptor binding assay scores improved by 19% in subfertile men (Roseff, 2002).

Other compounds

A study has been carried out evaluating the effects of combined conventional, oral antioxidants (*N*-acetyl-cysteine or vitamin A plus vitamin E) and essential fatty acids on sperm biology in an open prospective study including 27 infertile men. Sperm number increased in oligozoospermic men. Treatment significantly decreased ROS and 8-hydroxydeoxyguanine concentrations. Treatment improved the acrosome reaction rate, the proportion of PUFA in phospholipids and sperm membrane (Comhaire *et al.*, 2000).

Spermatozoa from 15 asthenozoospermic patients with high ROS concentrations were treated *in vitro* with pentoxifylline to establish its effect on ROS generation and sperm movement. These same 15 patients and 18 with asthenozoospermia whose spermatozoa did not produce ROS at steady state were treated with pentoxifylline at two different dosages (300 and 1200 mg daily) to verify its effect on ROS generation, sperm motion parameters and fertilizing ability *in vivo*. Pentoxifylline failed to decrease sperm ROS production and had no effect on sperm motility, sperm motion parameters and fertilizing ability. However, it improved motility and beat cross-frequency at high dosage (1200 mg daily) (Okada *et al.*, 1997).

Selenium supplementation alone did not seem to improve spermatozoon parameters: sperm count, motility and morphology (Iwanier and Zachara, 1995). Very recently, a randomized clinical trial explored the efficacy of selenium (200 μg) and/or *N*-acetyl-cysteine (600 mg) in 468 infertile men with idiopathic oligoasthenoteratozoospermia for 26 weeks. Selenium and *N*-acetyl-cysteine treatment resulted in a significant improvement of all sperm parameters. This was found to be associated with a positive correlation between the seminal plasma concentrations of selenium and *N*-acetyl-cysteine and semen parameters (Safarinejad and Safarinejad, 2009).

Some less common drugs have also been tested to verify their possible effects as antioxidants. Shao–Fu–Zhu–Yu–Tang has been described to have sperm antioxidant and anti-ageing properties (Yang *et al.*, 2003). Sperm quality and function improved with the intake of a combination of Zn and folic acid, or the antioxidant astaxanthin (Astacarox), or an energy-providing combination containing (acetyl)-carnitine (Proxeed). In addition, double-blind trials showed that the latter two substances increase spontaneous or intrauterine insemination-assisted conception rates (Lenzi *et al.*, 2003; Comhaire *et al.*, 2005). Moreover, extracts of the Peruvian plant *Lepidium meyenii* were shown to improve sperm morphology and concentration, respectively, in uncontrolled trials (Gonzales *et al.*, 2001).

Linseed (flaxseed) oil contains α-linolenic acid and lignans. The former corrects the deficient intake of omega-3 essential fatty acids, which is correlated with impaired sperm motility among subfertile men (Comhaire and Mahmoud, 2003).

Particularly, astaxanthin (Astacarox) seems to play a significant role in reducing ROS and inhibin B concentration and improving sperm linear velocity and pregnancy rate, from a double-blind randomized study (Comhaire *et al.*, 2005).

Sixty couples with severe male factor infertility were enrolled in a 3-month long, prospective randomized double-blind, placebo-controlled trial, to test Menevit antioxidant preparation prior to undergoing an IVF cycle. The group of patients treated with antioxidant showed a statistically significant enhancement of the pregnancy rate (38.5% of transferred embryos ensuing in a viable fetus at 13 weeks development) compared with the placebo group (16% pregnancy). No significant changes in oocyte fertilization rate or embryo quality were identified between the antioxidant and the placebo-treated group (Tremellen *et al.*, 2007).

Different concentrations of *Morindae officinalis* extract (0.25 and 0.5 g/ml) were shown to be significantly better than vitamin C in improving SOD vitality of sperm suspensions and in reducing MDA content. It was reported to play to a certain degree a protective role in the ROS-mediated injury of sperm membranes. Furthermore, the large dose (0.5 mg/ml) of *Morindae officinalis* especially protects sperm membrane function (Yang *et al.*, 2006). Recently, Omu and colleagues showed that Zn therapy is effective in reducing OS, sperm apoptosis and sperm DNA fragmentation index in asthenozoospermic men. Zn association with vitamin E and with vitamin E plus vitamin C did not show additional effects (Omu *et al.*, 2008).

Conclusion

Although many studies report positive effects of antioxidant treatment on semen quality, physicians needs to be aware that: (i) there is a lack of a well-defined therapeutic strategy during OS; (ii) the majority of the studies suffer from a lack of placebo-controlled, double-blind design which makes it difficult to reach a definitive conclusion, as also reported by Sigman and Patel (2008); (iii) an early diagnosis of infertility is necessary to avoid progressive OS-induced damage that may reach an irreversible state; and (iv) a correct evaluation of the ROS concentrations or of lipid peroxidation, such as MDA, is useful to estimate the degree of OS, which may assist clinicians both to elucidate the role of OS on the fertility condition and to evaluate the effects of the antioxidant treatment.

From the up-to-date information, it emerges that no single antioxidant is able to enhance fertilizing capability in infertile men and a combination of compounds, at an appropriate dosage, may be a possible better approach. Taking into account the pros and the cons of antioxidant administration to infertile men (**Table 2**), the potential advantages that such treatment offers cannot be ignored. Antioxidant therapy has been available for a long time and remains a very important aspect of preventive medicine. This needs to be communicated to all those working in the field of human reproductive medicine. An appropriate diet supplying an appropriate amount of antioxidants and a healthy lifestyle

Table 2. Advantages and disadvantages of antioxidant therapy.

Advantages
Improved sperm motility (including in asthenozoospermia), improved kinetic parameters and hyperactivation; post-freezing–thawing motility is prolonged; asthenozoospermia after *in vitro* supplementation is counteracted; WBC-mediated sperm motility loss after *in vitro* supplementation is reduced
Sperm concentration and total sperm counts in patients with asthenozoospermia or oligoasthenozoospermia is increased
Percentage of abnormal spermatozoa is decreased
Sperm viability is improved
Sperm acrosome reaction rate is increased
Oocyte penetration and fertilization rate: the decreased oocyte penetration rate induced by ROS is counteracted; oocyte fertilization is improved; fertility rate is increased
Fertilization rate per cycle is improved
Fertilizing potential of fertile normozoospermic men with low fertilization rates in previous IVF cycles is increased
Pregnancy rate is improved (very few studies)
Generation of ROS is suppressed
Intracellular content of cAMP and cGMP is increased
Proportion of PUFA in phospholipids and sperm membranes is reduced
Sperm DNA fragmentation is reduced
An anti-apoptotic effect in a variety of cell cultures has been demonstrated
Disadvantages
Lack of placebo-controlled, double-blind design in the majority of the studies
Diagnostic strategy: lack of well-defined markers to estimate the oxidative stress before and after any antioxidant treatment; lack of identified markers useful to evaluate which oxidative stress-induced damage will benefit from the antioxidant treatment
Therapeutic strategy: lack of knowledge in the following areas – the different combinations of antioxidant compounds, the dosages of the various antioxidants and the length of treatment. Also, commercial supplements for fertility often contain low antioxidant doses
Patient's clinical condition: patients often present with fertility problems many years after the beginning of their pathology. In these cases, the progressive oxidative stress-induced damage can become irreversible, consequently antioxidant treatment is ineffective; the efficacy of different antioxidant therapies differs in different male reproductive pathologies; contemporary presence of pro-oxidant factors (e.g. germs and WBC, often difficult to eradicate); effectiveness of antioxidant is often linked to the cause of the ROS production
Dose-dependent action: at some concentrations a pro-oxidant effect is present resulting in an abrupt fall in sperm motility and viability and an increase in liporoxidation. Certain antioxidants may have embryo-toxic and teratogenic effects. High doses of vitamin C may cause kidney stone formation
Pregnancy as a parameter of efficacy of treatment has been reported in few studies

PUFA = polyunsaturated fatty acids; ROS = reactive oxygen species; WBC = white blood cell.

(especially cessation of cigarette smoking) are certainly additional aspects that need to be taken into account.

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