

## Review

# Role of antioxidants in treatment of male infertility: an overview of the literature



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## Abstract

Seminal oxidative stress in the male reproductive tract is known to result in peroxidative damage of the sperm plasma membrane and loss of its DNA integrity. Normally, a balance exists between concentrations of reactive oxygen species and antioxidant scavenging systems. One of the rational strategies to counteract the oxidative stress is to increase the scavenging capacity of seminal plasma. Numerous studies have evaluated the efficacy of antioxidants in male infertility. In this review, the results of different studies conducted have been analysed, and the evidence available to date is provided. It was found that although many clinical trials have demonstrated the beneficial effects of antioxidants in selected cases of male infertility, some studies failed to demonstrate the same benefit. The majority of the studies suffer from a lack of placebo-controlled, double-blind design, making it difficult to reach a definite conclusion. In addition, investigators have used different antioxidants in different combinations and dosages for varying durations. Pregnancy, the most relevant outcome parameter of fertility, was reported in only a few studies. Most studies failed to examine the effect of antioxidants on a specific group of infertile patients with high oxidative stress. Multicentre, double-blind studies with statistically accepted sample size are still needed to provide conclusive evidence on the benefit of antioxidants as a treatment modality for patients with male infertility.

**Keywords:** antioxidants, assisted reproduction, male infertility, reactive oxygen species, spermatozoa

## Introduction

Infertility affects approximately 15% of all couples trying to conceive, and male factor is the sole or contributing factor in roughly half of these cases (Sharlip *et al.*, 2002). A variety of medications have been developed in an attempt to improve the sperm quality and in turn modify the male fertility potential (Kamischke and Nieschlag, 1999; Siddiq and Sigman, 2002; Comhaire and Mahmoud, 2003). In the era of evidence-based medicine, specific management of infertility should be based on identifying reversible causes of infertility and treating them with suitable medications. However, this may constitute a challenge, since in spite of extensive research, no identifiable cause can be found in over 25% of infertile males (March and Isidori, 2002).

Recently, oxidative stress (OS) has become the focus of interest as a potential cause of male infertility (Sharma and Agarwal, 1996; Pasqualotto *et al.*, 2000a, 2001; Gil-Guzman *et al.*, 2001; Aitken and Krausz, 2001; Agarwal and Saleh, 2002; Saleh and Agarwal, 2002; Agarwal and Said, 2003; Aitken *et al.*, 2003; Wang *et al.*, 2003a). Under physiological conditions, spermatozoa produce small amounts of reactive oxygen species (ROS), which are needed for capacitation, acrosome reaction and fertilization (Griveau and Le Lannou, 1997). However, excessive amounts of ROS produced by leukocytes and immature spermatozoa can cause damage to the normal spermatozoa by inducing lipid peroxidation and DNA damage (Aitken *et al.*, 1998; Ollero *et al.*, 2001; Alvarez *et al.*, 2002; Saleh *et al.*, 2002a,b, 2003; Agarwal *et al.*, 2003; Wang *et al.*, 2003b; Moustafa *et al.*, 2004). Normally, an equilibrium exists between ROS production and antioxidant scavenging activities in the male reproductive tract. However,

the production of excessive amounts of ROS in semen may overwhelm the antioxidant defence mechanisms of spermatozoa and seminal plasma, and cause OS (Sikka *et al.*, 1995; Sikka, 2004). OS status of an individual can be identified by measuring the ROS concentrations and antioxidants (Shekarriz *et al.*, 1995a; Sharma and Agarwal, 1996; Esfandiari *et al.*, 2003). ROS concentrations are usually measured by chemiluminescence (Shekarriz *et al.*, 1995b; Kobayashi *et al.*, 2001), while total antioxidant capacity is measured by enhanced chemiluminescence assay or colorimetric assay (Sharma *et al.*, 1999; Said *et al.*, 2003).

The seminal plasma is well endowed with an array of antioxidants that act as free radical scavengers to protect spermatozoa against OS (Smith *et al.*, 1996; Agarwal and Saleh, 2002). This defence mechanism compensates for the loss of sperm cytoplasmic enzymes occurring when the cytoplasm is extruded during spermiation, which in turn, diminishes endogenous repair mechanisms and enzymatic defences. Seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase. In addition, it contains a variety of non-enzymatic antioxidants such as vitamin C (ascorbic acid), vitamin E ( $\alpha$ -tocopherol), pyruvate, glutathione, and carnitine (Saleh and Agarwal, 2002).

High concentrations of ROS may be detected in the semen of 30–80% of infertile men (unpublished observation) and in almost all spinal cord injury patients (de Lamirande *et al.*, 1995; Padron *et al.*, 1997). In view of this, rational strategies with the goal of reducing concentrations of OS should be effective in the treatment of male infertility. Initially, clinicians should identify and treat the cause for increased ROS production, for example reproductive tract infections, smoking, and varicocele (Hendin *et al.*, 1999; Kolettis *et al.*, 1999; Pasqualotto *et al.*, 2000b; Sharma *et al.*, 2001; Saleh *et al.*, 2002c). Following this, augmentation of the scavenging capacity of the seminal plasma by supplementation with antioxidants should be considered. Unfortunately, the results of clinical trials using antioxidants in the treatment of infertile men remain an issue of controversy mainly due to too many variables in these studies (Ford and Whittington, 1998; Geva *et al.*, 1998; Lenzi *et al.*, 1998; Martin-Du Pan and Sakkas, 1998; Tarin *et al.*, 1998). Investigators have used different antioxidants in different combinations and dosages for varying durations. In addition, they used different outcome parameters of fertility to assess the efficiency of these antioxidants. However, even statistically significant improvements in semen characteristics do not always translate into clinical benefit; this can only be done by establishing a relevant clinical end-point, ideally in-vivo fecundity, which must show statistically significant improvement after controlling for pertinent clinical factors such as female factors. In the following sections, an attempt will be made to focus on the available evidence on the role of antioxidants in male infertility and provide a systematic review on the efficacy of antioxidants.

## Profile of studies on antioxidants

A meticulous search of the literature was conducted, searching the MEDLINE database using PubMed with different keywords (antioxidants, male infertility, spermatozoa). The references cited in the studies identified by a PubMed search

were reviewed. Using the English language literature, studies on humans related to male infertility and antioxidants were identified.

In the literature search, a total of 57 studies was found; 10 studies were randomized controlled trials, 16 controlled and 31 uncontrolled studies. There were 26 in-vivo and 33 in-vitro interventional studies. Forty-eight studies evaluated a single antioxidant agent and 11 others evaluated combinations of multiple antioxidants. ROS was measured in 25 studies, lipid peroxidation in 12 studies, DNA damage was evaluated in nine studies, sperm function tests in 12 and sperm parameters in 52 studies. Effect of antioxidants on the assisted reproduction outcome and natural pregnancy were reported by fertilization and pregnancy rates in 15 studies.

## Effects of antioxidants on semen quality

### In-vitro studies: positive effect

Vitamin E is a major chain breaking antioxidant in the sperm plasma membranes (Bolle *et al.*, 2002). Several in-vitro studies have reported its effectiveness in protecting sperm motility and morphology. Since vitamin E (10 mmol/l) maximally preserved sperm motility by suppressing lipid peroxidation (Aitken and Clarkson, 1988; Aitken *et al.*, 1989), it appears to have a dose-dependent protective effect. Cryopreservation and thawing procedures are associated with a significant reduction in sperm motility induced by oxidative stress (Critser *et al.*, 1988; Mazilli *et al.*, 1995). The employment of vitamin E (10 mmol/l) along with cryoprotectants during cryopreservation is capable of preserving sperm motility more efficiently than cryoprotectant alone (Askari *et al.*, 1994).

Vitamin C (ascorbate) is the principal antioxidant in seminal plasma of fertile men, contributing up to 65% of its total chain-breaking antioxidant capacity. Concentration of ascorbate in seminal plasma is 10 times greater than in blood plasma (364 versus 40  $\mu$ mol/l) (Lewis *et al.*, 1997). Verma and Kanwar (1998) demonstrated the dose dependant preservation of sperm motility by vitamin C. Motility was highest after 6 h incubation in 800  $\mu$ mol/l vitamin C ( $P < 0.001$ ). However, reduction in motility was initiated with concentrations  $>1000$   $\mu$ mol/l (Verma and Kanwar, 1998). This may be attributed to the fact that at higher doses, vitamin C may paradoxically increase ROS production in presence of catalytic cations such as iron.

Glutathione (GSH) appears also to have a protective effect on sperm motility. In leukocytospermic samples, higher recovery of motile spermatozoa was observed with GSH compared with Tyrode's solution after Percoll preparation and after 24-h incubation (Parinaud *et al.*, 1997). Similarly, Oeda *et al.* evaluated the antioxidant effect of N-acetyl-L-cysteine (NAC) on motility; a significant improvement was observed in sperm motility after 2 h of incubation at a dose of 1.0 mg/ml (Oeda *et al.*, 1997).

Albumin also acts as an antioxidant (0.5 and 1%), as it specifically reacts against peroxy radicals and prevents the

propagation of peroxidative damage in spermatozoa (Hong *et al.*, 1994; Twigg *et al.*, 1998). Use of albumin in sperm preparation media resulted in significant improvement in motility and viability compared with Percoll (Armstrong *et al.*, 1998). Other antioxidants that preserve sperm motility include coenzyme Q10 (50  $\mu\text{mol/l}$ ) (Lewin and Lavon, 1997), hypotaurine (10  $\text{mmol/l}$ ), and catalase (2600 IU) (Baker *et al.*, 1996).

Superoxide dismutase (SOD) and catalase are enzymatic antioxidants that protect spermatozoa from superoxide anion and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The addition of exogenous SOD (400 IU/ml) to the sperm suspension significantly reduces the loss of motility ( $P < 0.005$ ) and malondialdehyde (MDA) concentration (Kobayashi *et al.*, 1991). The study by Gagnon *et al.* refers to  $\text{H}_2\text{O}_2$  as a primary toxic ROS responsible for most damage done to spermatozoa, and demonstrates that the addition of catalase (0.008  $\text{mg/ml}$ ) to sperm suspension offers complete protection (Gagnon *et al.*, 1991). Several other studies supported the role of catalase and SOD against sperm-intracellular (mitochondrial and plasma membrane) and -extracellular (leukocytes) ROS and their beneficial effects on sperm motility (Kovalski *et al.*, 1992; Aitken *et al.*, 1993, 1995; Griveau and Le Lannou, 1994) and tail-beat frequency (Hong *et al.*, 1994).

### In-vitro studies: no effect

Vitamin C has the ability to promote the release of transition metals (iron and copper) from proteins to redox cycle to form oxygen radicals (Gutteridge, 1994), and therefore can act as a pro-oxidant when added in isolation. Higher concentrations of vitamin C ( $>20 \mu\text{mol/l}$ ) are not protective against  $\text{H}_2\text{O}_2$ -induced peroxidative damage of motility; instead, they increase damage in both normozoospermic and asthenozoospermic patients (Donnelly *et al.*, 1999a). Another study showed that vitamin C (10  $\text{mmol/l}$ ) along with TEST yolk buffer failed to reduce the loss of motility in cryopreserved semen samples (Askari *et al.*, 1994). Similar results were obtained with vitamin E at concentrations of 40 and 60  $\mu\text{mol/l}$  (Donnelly *et al.*, 1999a). In support, it was demonstrated that the net hydroxyl radical-absorbing capacity of vitamin E increased between the concentration range of 0.1–20  $\mu\text{mol/l}$  (i.e. lower than the normal range for human serum). At higher concentrations (20–400  $\mu\text{mol/l}$ ), the hydroxyl radical-absorbing capacity actually decreased, and at final concentrations of  $>1000 \mu\text{mol/l}$ , vitamin E acted as an oxidation stimulator rather than an antioxidant (Cao *et al.*, 1993). Another study revealed that vitamins E and C did not improve the motility loss by ROS released from leukocytes compared with other antioxidants (GSH, NAC, hypotaurine, and catalase) (Baker *et al.*, 1996).

Sperm preparation for assisted reproductive techniques involves several steps of centrifugation and prolonged incubation, which induces OS and subsequently affects sperm motility and assisted reproduction outcome. Thus, although there is a strong body of evidence supporting the use of antioxidants in assisted reproduction media as supported by the above studies, the dosage and combination of antioxidants to be added to the assisted reproduction media should be cautiously selected, as excessive amounts of antioxidants may act paradoxically to damage spermatozoa. Use of antioxidants

in cryopreservation media may be advised to preserve motility. Various antioxidants used during in-vitro experiments and their mechanism of action, dosage and their effect are illustrated in **Table 1**.

### In-vivo studies: positive effect

The beneficial effect of vitamin C oral administration on sperm quality has been documented in smokers. Significant improvement was seen in serum and seminal plasma vitamin C concentrations when doses of 200 and 1000  $\text{mg}$  were administered ( $P < 0.01$ ,  $P < 0.001$ , respectively). A greater improvement in sperm quality was observed in the 1000  $\text{mg}$ -treated group (significant improvement in concentration, morphology and viability) than in the 200  $\text{mg}$ -treated group. A significant positive correlation was observed between serum, seminal plasma vitamin C concentrations and sperm quality ( $r = 0.56$ – $0.87$  for different sperm parameters) (Dawson *et al.*, 1992).

A randomized double-blind placebo controlled trial reported that treatment with oral vitamin E (300  $\text{mg}$  twice a day) significantly improved sperm motility ( $P < 0.001$ ) in 60% of asthenozoospermic patients, while only 11% of patients in the placebo group showed improvement in motility (Suleiman *et al.*, 1996). In addition, the combination of vitamin E (200  $\text{mg/day}$ ), vitamin C (200  $\text{mg}$ ), and GSH (400  $\text{mg}$ ) in infertile patients for 2 months resulted in significant improvement in sperm concentration ( $P < 0.05$ ) (Kodama *et al.*, 1997). Another combination of vitamin E (400  $\text{mg/day}$ ) and selenium (100  $\mu\text{g/day}$  for 1 month and 200  $\mu\text{g/day}$  for the next 5 months) in oligoasthenoteratozoospermic (OAT) patients resulted in an increase in seminal plasma concentrations of selenium and vitamin E along with significant improvement in sperm motility (12.1–31.1%), viability (34.8–62.7%) and morphology (33.6–66.2%) (Vezina *et al.*, 1996). Similarly, improvement in motility was observed in a recent study using vitamin E (400  $\text{mg}$ ) and selenium (225  $\mu\text{g}$ ) supplementation for 3 months in a group of volunteers and infertile patients (Keskes-Ammar *et al.*, 2003).

Another prospective study evaluated the use of vitamin E combinations in 27 infertile patients. Antioxidants (180  $\text{mg}$  vitamin E, NAC 600  $\text{mg/day}$  or  $\beta$ -carotene 30  $\text{mg/day}$ ) were administered along with conventional treatment and essential fatty acids for 6 months. Significant improvement in sperm concentration without any change in motility and morphology was seen in oligozoospermic patients (Comhaire *et al.*, 2000).

Studies by Lenzi *et al.* demonstrated that therapeutic action of GSH is due to its protective effect on lipid components of the cell membrane (Lenzi *et al.*, 1992, 1993, 1994). When infertile patients with high production of ROS were treated with GSH (600  $\text{mg}$ ), a positive effect on sperm motility ( $P < 0.003$ ), in particular on the percentage of forward progression ( $P < 0.001$ ), and on sperm morphology was observed after 30 days of treatment. These results indicate the action of GSH as a free radical scavenger, which reduces the lipid peroxidative process.

The alteration of carnitine and acetylcarnitine ratio in seminal plasma of asthenozoospermic samples, rationalized their use in selected cases of male infertility (Bartellini *et al.*, 1987). In a

**Table 1.** In-vitro effects of antioxidants.

<i>Antioxidant</i>	<i>Mechanism of action</i>	<i>Dose</i>	<i>In-vitro effect</i>	<i>Studies</i>
Vitamin E	Major chain breaking antioxidant in membranes, directly neutralizes superoxide anion (O <sub>2</sub> <sup>-•</sup> ), hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> ) and hydroxyl (OH <sup>•</sup> ) radical	10 mmol/l	Prevents motility reduction and reduces lipid peroxidation	Aitken and Clarkson, 1988; Aitken <i>et al.</i> , 1989; Askari, 1994; Verma and Kanwar, 1998
		40 and 60 µmol/l 1–100 µmol/l	Reduces H <sub>2</sub> O <sub>2</sub> -induced ROS Reduction in H <sub>2</sub> O <sub>2</sub> -induced DNA damage	Donnelly <i>et al.</i> , 1999a Sierens <i>et al.</i> , 2002
Vitamin C	Chain breaking antioxidant, competitively protects the lipoproteins from peroxy radicals and also recycles vitamin E	800 µmol/l 40 and 60 µmol/l	Prevents motility reduction Reduces H <sub>2</sub> O <sub>2</sub> -induced ROS	Verma and Kanwar, 1998 Donnelly <i>et al.</i> , 1999b
		100–200 µmol/l 300 and 600 µmol/l	Reduces H <sub>2</sub> O <sub>2</sub> -induced DNA damage Reduces H <sub>2</sub> O <sub>2</sub> -induced DNA damage	Sierens <i>et al.</i> , 2002 Donnelly <i>et al.</i> , 1999b
Glutathione	Neutralizes O <sub>2</sub> <sup>-•</sup> , reduced glutathione metabolizes H <sub>2</sub> O <sub>2</sub> and OH <sup>•</sup>	10 mmol/l	Reduces DNA damage induced by ROS	Lopes <i>et al.</i> , 1998; Griveau and Le Lannou, 1994
		–	Prevents motility reduction in leukocytospermic samples	Parinaud <i>et al.</i> , 1997
		5–10 mmol/l 1–10 mmol/l	Reverses FeSO <sub>4</sub> -inhibited tail-beat frequency Reduces PMA-induced ROS and protects motility	Hong <i>et al.</i> , 1994 Baker <i>et al.</i> , 1996
N-Acetyl-L-cysteine	May act as a precursor of glutathione and thus facilitates its biogenesis	0.1–5 mg/ml 1–10 mmol/l	Prevents motility reduction Reduces ROS production and improves motility and motion kinetics	Oeda <i>et al.</i> , 1997 Baker <i>et al.</i> , 1996
		0.1 mmol/l	Reduces DNA damage	Lopes <i>et al.</i> , 1998
Pentoxifylline	Phosphodiesterase inhibitor and acts as motility stimulant, in addition reduces the superoxide anion release	3.6–7.2 mmol/l	Reduces ROS production and lipid peroxidation	McKinney <i>et al.</i> , 1996
		10 mmol/l 1–10 mmol/l	Reduces superoxide anion production Prevents reduction of curvilinear velocity, beat-cross frequency and reduces ROS production and lipid peroxidation	Gavella <i>et al.</i> , 1991; Gavella and Lipovac, 1992 Okada <i>et al.</i> , 1997
Superoxide dismutase (SOD)	Neutralizes intra- and extracellular superoxide anion	87.5–500 IU/ml	Reduces loss of motility and lipid peroxidation	Kobayashi <i>et al.</i> , 1991; Aitken <i>et al.</i> , 1993, 1995; Griveau and Le Lannou, 1994; Lopes <i>et al.</i> , 1998
Catalase	Neutralizes intra- and extracellular hydrogen peroxide	1 mg/ml	Reduces loss of motility	Kovalski <i>et al.</i> , 1992
		0.008–0.1 mg/ml, 50–2000 IU/ml	Reduces loss of motility and lipid peroxidation and DNA damage	Lopes <i>et al.</i> , 1998; Kovalski <i>et al.</i> , 1992; Griveau and Le Lannou, 1994
Coenzyme Q <sub>10</sub>	Energy promoting agent, may act as an antioxidant by reducing superoxide anion generation	50 µmol/l	Improves sperm motility	Lewin and Lavon, 1997

O<sub>2</sub><sup>-•</sup> = superoxide anion; H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide; OH<sup>•</sup> = hydroxyl radical; ROS = reactive oxygen species; CASA = computer assisted semen analyser.

multicentre clinical trial, in-vivo administration of L-carnitine (3 g/day for 4 months) in 134 asthenozoospermic patients produced a significant improvement in motility ( $P < 0.001$ ), rapid linear progression ( $P < 0.001$ ), mean velocity ( $P < 0.001$ ), and linearity index ( $P < 0.001$ ) (Costa *et al.*, 1994).

Vicari and Calogero (2001) evaluated the effects of carnitines in patients with prostatic-vesiculo-epididymitis (PVE) in the presence or absence of pro-oxidant factors. Abacterial PVE infertile patients with normal (group A,  $n = 34$ ) or abnormal (group B,  $n = 20$ ) seminal white blood cell (WBC) concentrations received carnitines (L-carnitine 1 g and acetylcarnitine 0.5 g twice/day) for 3 months, followed by a washout period of 3 months. Carnitines increased sperm forward progression and viability in group A patients, along with significant reduction in ROS production, which persisted during washout. On the other hand, carnitines increased only the percentage of viable spermatozoa in group B patients. These results were supported by an open prospective randomized trial in PVE patients which demonstrated that anti-inflammatory treatment followed by carnitines offers the best chances for improvement in the sperm motility and viability (Vicari *et al.*, 2002). Further information on carnitines and male infertility can be obtained from a recent review (Agarwal and Said, 2004).

Oral pentoxifylline at a dosage of 300 mg daily administered four times had no effect on sperm motility or sperm motion parameters. However, it increased motility and beat-cross frequency at a high dosage (1200 mg daily) (Okada *et al.*, 1997). A randomized double-blind placebo controlled study examined men with reduced motility for a 3-month period after oral supplementation of selenium alone (100 µg/day) and selenium in combination with vitamin A (1 mg), C (10 mg) and D (15 mg). Sperm motility was significantly improved in the treatment group ( $P < 0.02$ ) (Scott *et al.*, 1998).

### In-vivo studies: no effect

A randomized controlled trial studied in-vivo effect of vitamin C (200 mg/day for 6 months with a 3-month follow-up period) (Abel *et al.*, 1982). In this trial, the dose of vitamin C tested was very minimal and may be the reason for no improvement in sperm parameters and pregnancy rate. A similar study assessed treatment of infertile men with vitamin C (200 mg/day for 6 months with a 3-month follow-up period) found no improvement in sperm parameters after treatment (Hargreave *et al.*, 1984).

The first double-blind randomized placebo crossover controlled trial by Kessopoulou evaluated the effect of oral administration of vitamin E (600 mg/day for 3 months) on semen quality in 30 healthy men with high levels of ROS generation. There was an increase in the blood serum vitamin E concentrations after treatment ( $P = 0.04$ ), while no significant change in sperm parameters was observed (Kessopoulou *et al.*, 1995). The lack of improvement in semen parameters may be because of insufficient concentrations of antioxidants attained in seminal plasma. Vitamin E may have a spectrum of effects depending on its concentration in seminal plasma. Similar findings were observed using 600 mg, 800 mg, and 1200 mg vitamin E daily for 3 weeks (Moilanen and

Hovatta, 1995). Although significant improvement ( $P < 0.05$ ) in median blood and seminal plasma concentrations was achieved with 800 mg/day oral therapy, no improvement in semen parameters was observed. These results were supported by another prospective study, in which the administration of 200 mg of vitamin E did not improve the morphology of the spermatozoa (Geva *et al.*, 1996). However, it is important to note that the dose administered was very low for an antioxidant effect to occur.

Therefore, it appears that both vitamins C and E do not always have a positive effect on sperm parameters. Their combination (1000 mg vitamin C and 800 mg vitamin E) for 56 days did not improve conventional semen parameters in infertile men with oligo-asthenozoospermia. A possible explanation for these controversial results may be due to the short duration of therapy and patient selection criteria (Rolf *et al.*, 1999).

Even although several studies have demonstrated the positive effects of in-vivo antioxidants on semen quality, others have failed to show such an effect. The reasons may be due to very low in-vivo dose of antioxidants (Abel *et al.*, 1982; Hargreave *et al.*, 1984; Geva *et al.*, 1996) or short duration of treatment (Moilanen and Hovatta, 1995; Rolf *et al.*, 1999). In-vivo supplementation should reach specific concentrations in seminal plasma in order to act effectively against OS. Various dosages and durations of antioxidant treatment should be evaluated and related to outcome parameters such as semen quality parameters along with pregnancy outcome.

## Effects of antioxidants on lipid peroxidation

### In-vitro studies: positive effect

The increase in sperm membrane lipid peroxidation decreases the motility of the spermatozoa (Jones *et al.*, 1978). This can be prevented effectively by the antioxidants in the seminal plasma (Jones *et al.*, 1979). The exogenous addition of the enzyme SOD (400 IU/ml) prevents lipid peroxidation ( $P < 0.01$ ) (Kobayashi *et al.*, 1991). Variable concentrations of vitamin E (800 µmol/l, 10 mmol/l) proved to be protective against lipid peroxidation (Aitken *et al.*, 1989; Verma and Kanwar, 1998). In addition, pentoxifylline at a dosage of 3.6 mmol/l and 7.2 mmol/l was able to reduce lipid peroxidation significantly in asthenozoospermic men (McKinney *et al.*, 1996).

### In-vitro studies: no effects

An interesting study demonstrated that lipid peroxidation induced by NADPH could not be prevented by albumin, SOD (100, 250, 500 IU), catalase (200, 500, 2000 U), vitamin E (mmol/l) and pentoxifylline (3.6 mmol/l) (Twigg *et al.*, 1998). Albumin (0.3, 1.0, 3.0, 10.0%) may only be effective when there is increased intracellular ROS production. Thus, it appears that effectiveness of antioxidants depends on the source of the OS.

## In-vivo studies: positive effects

In-vivo supplementation of GSH for 60 days in 10 infertile patients significantly reduced lipid peroxidation potential ( $P < 0.001$ ) along with improvement in sperm membrane characteristics (Lenzi *et al.*, 1994). GSH acts as a free radical scavenger in the epididymis, and thus may reduce the lipid peroxidative process. Significant reduction of lipid peroxidation ( $P < 0.001$ ) in vitamin E (300 mg twice a day for 6 months) treated asthenozoospermic patients was observed (Suleiman *et al.*, 1996). Similarly, 200 mg daily for 1 month of oral vitamin E significantly reduced lipid peroxidation and significantly improved fertilization rate per IVF cycle ( $P < 0.05$ ) in 15 normozoospermic males with low fertilization rates (Geva *et al.*, 1996). When vitamin E (200 mg) in combination with vitamin C (200 mg) and GSH (400 mg) were administered orally for 2 months, significantly improved serum concentrations of vitamin E and vitamin C along with significant reduction in lipid peroxidation ( $P < 0.01$ ) was observed in OAT patients (Kodama *et al.*, 1997). A recent open randomized trial has shown that 3 months' supplementation of vitamin E and selenium produced a significant decrease in MDA concentrations (Keskes-Ammar *et al.*, 2003).

## Effects of antioxidants on concentrations of ROS

### In-vitro studies: positive effect

The addition of different concentrations of vitamin C (300 and 600  $\mu\text{mol/l}$ ) and vitamin E (40 and 60  $\mu\text{mol/l}$ ) to sperm preparation medium significantly reduced ( $P < 0.005$ )  $\text{H}_2\text{O}_2$ -induced ROS in all concentrations (Donnelly *et al.*, 1999b, 2000). The dose-dependent action of NAC (0.1, 1 and 5 mg/ml) in reducing the concentration of ROS was also evaluated (Oeda, 1997). Incubation with NAC (1 mg/ml) significantly reduced ROS concentrations after 20 and 40 min ( $P < 0.01$  and  $P < 0.001$ ). This reduction was greater in subjects with high ROS ( $P < 0.0001$ ) than in subjects with low ROS.

Gavella *et al.* demonstrated the in-vitro effects of pentoxifylline on superoxide anion generation (Gavella *et al.*, 1991; Gavella and Lipovac, 1992). Superoxide anion release stimulated by phorbol myristate acetate (PMA) was reduced by 29–72% following the addition of 10 mmol/l pentoxifylline ( $P < 0.001$ ). Other studies also showed that pentoxifylline is capable of reducing spermatozoal generation of ROS and subsequent lipid peroxidation in asthenozoospermic men (McKinney *et al.*, 1996; Okada *et al.*, 1997).

### In-vivo studies: positive effect

The combination therapy of vitamin E with NAC or vitamins A plus E and essential fatty acids significantly reduced ROS ( $P < 0.01$ ) (Comhaire *et al.*, 2000).

Carnitines (L-carnitine 1 g and acetylcarnitine 0.5 g), when given twice per day for 3 months followed by a wash-out period of 3 months in patients with PVE (leukocytospermic and aleukocytospermic), resulted in a significant reduction in ROS production, which persisted during washout (Vicari and Calogero, 2001). In support, treatment with carnitines was

fully effective in reducing ROS production when administered after a 3-month course of non-steroidal anti-inflammatory drugs (NSAID) and less effective when given concomitantly with NSAID (Vicari *et al.*, 2002). However, no control group was included in this study.

### In-vivo studies: no effect

Treatment of patients with high levels of ROS generation in semen with 600 mg/day of vitamin E for 3 months resulted in an increase in the serum vitamin E concentrations after treatment ( $P = 0.04$ ) with improvement in zona-binding ratio ( $P = 0.004$ ); however, there was no significant change in sperm parameters and ROS generation (Kessopoulou *et al.*, 1995). The lack of improvement in ROS concentrations may be due to vitamin E action as a chain-breaking compound rather than a scavenger of ROS.

## Effects of antioxidants on sperm DNA damage

### In-vitro studies: positive effect

Sperm preparation techniques involve repeated high-speed centrifugation and the isolation of spermatozoa from the protective antioxidant environment provided by seminal plasma. This has been shown to damage sperm DNA via mechanisms that are mediated by the enhanced generation of ROS (Zalata *et al.*, 1995). Pretreatment with antioxidants [NAC (0.01 mmol/l), catalase (500 IU/ml), reduced GSH (10 mmol/l) and hypotaurine (10 mmol/l)] significantly reduces DNA damage ( $P < 0.04$ ) induced by ROS generation in sperm samples of infertile patients (Lopes *et al.*, 1998). In addition, albumin in doses from 0.3 to 10% may contribute to the protection of DNA and prevention of DNA damage by neutralizing peroxides produced during lipid peroxidation (Twigg *et al.*, 1998). A recent study reported that addition of antioxidants that target mitochondria (synthetic SOD/catalase, and N-tert-butyl hydroxylamine) can decrease release of chromatin from spermatozoa maintained in IVF media (Lamond *et al.*, 2003).

Individual protective roles of vitamin E and vitamin C on sperm DNA damage induced by  $\text{H}_2\text{O}_2$  were demonstrated in both normozoospermic and asthenozoospermic samples (Donnelly *et al.*, 1999b). When sperm suspension was incubated at 300 and 600  $\mu\text{mol/l}$  of vitamin C, both concentrations provided complete protection against  $\text{H}_2\text{O}_2$  induced DNA damage ( $P < 0.005$ ). Similar dose dependant protection was observed with vitamin E ( $P < 0.005$ ). However, when spermatozoa were incubated with a combination of vitamin C and vitamin E, DNA damage was exacerbated. This was probably caused by these vitamins acting as pro-oxidants. Similar toxicity was also reported using a combination of vitamins C and E on sperm suspension (Hughes *et al.*, 1998).

### In-vivo studies: positive effect

Oral administration of 200 mg of vitamin C, 200 mg of vitamin E and 400 mg of GSH for 2 months significantly improved serum concentrations of antioxidants and relatively decreased sperm DNA damage ( $P < 0.05$ ) (Kodama *et al.*, 1997). In addition, NAC and/or a mixture of essential fatty acids and

natural vitamins A and E were capable of reducing sperm OS-induced DNA damage as manifested by a decrease in 8-hydroxydeoxyguanosine (8-OH-dG) (Comhaire *et al.*, 2000).

The examination of the chromatin structure appears to be a useful tool for investigation of semen preservation. Vitamins C and E work synergistically to protect against lipid peroxidation, with vitamin C recycling vitamin E and allowing it to again function as a free radical chain breaker (Buettner, 1993). However, from the above results, it may be concluded that a tightly regulated optimal concentration exists for these two vitamins in combination, outside of which there is no benefit or protective effect against free radicals (Sweetman *et al.*, 1997).

## Effects of antioxidants on pregnancy outcome

### In-vivo studies: positive effect

Treatment with oral vitamin E and a combination of vitamin E with NAC or vitamins A, E and essential fatty acids significantly improves pregnancy rate in asthenozoospermic patients (Suleiman *et al.*, 1996; Comhaire *et al.*, 2000). Several studies have demonstrated the ability of carnitines to increase the chances of a couple to become pregnant (Vicari and Calogero, 2001; Vicari *et al.*, 2002; Lenzi *et al.*, 2003). A randomized double-blind placebo-controlled trial reported that treatment of oral vitamin E (300 mg twice a day) significantly improved pregnancy rate (21%; 11/52) of the asthenozoospermic patients, while no pregnancies were reported in the placebo group (Suleiman *et al.*, 1996). A prospective study by Comhaire *et al.* using the combination therapy of vitamin E with NAC or vitamins A, E and essential fatty acids in infertile men significantly improved overall pregnancy rate (Comhaire *et al.*, 2000). Smoking history appears to play a role in determining the response to antioxidant therapy. The per month pregnancy rate was higher in partners of ex-smokers (7.15%) than in non-smokers (1.6%).

The effects of L-carnitine 1 g and acetylcarnitine 0.5 g twice a day were evaluated in patients with PVE for 3 months followed by a washout period of 3 months. Within 3 months after the discontinuation of carnitines, the rate of spontaneous pregnancy in non-leukocytospermic patients was significantly higher [11.7% (4/34)] than the leukocytospermic group (0%) (Vicari and Calogero, 2001). These results demonstrate that treatment with carnitines may be effective in patients with abacterial PVE when seminal leukocyte concentration is normal. Another open prospective randomized trial demonstrated that anti-inflammatory treatment followed by carnitine is the best strategy in these PVE patients. This group showed higher pregnancy rate (23.1%,  $P < 0.01$ ) over the other groups (Vicari *et al.*, 2002). Finally, a placebo-controlled, double-blind, crossover trial was conducted on 100 infertile patients who underwent L-carnitine therapy 2 g/day or placebo for 2 months of washout, 2 months of therapy/placebo, 2 months of washout, and 2 months of placebo/therapy (Lenzi *et al.*, 2003). Eight pregnancies were achieved in the observation period during the L-carnitine therapy period.

In a randomized controlled trial by Scott *et al.*, 69 OAT patients were supplemented with placebo, selenium alone or selenium plus vitamins A, C and E daily for 3 months. Selenium treatment improved pregnancy rate and 11% achieved paternity in the treatment group, in contrast to none in the placebo group. This trial shows that selenium supplementation in subfertile men with low selenium status can improve sperm motility and the chance of successful conception (Scott *et al.*, 1998).

Finally, it is of interest to note that the improvement in the fertilization rate is not always accompanied by a concomitant improvement in any of the sperm parameters. Treatment of subfertile patients with low fertilization rates after ICSI with oral coenzyme Q<sub>10</sub> 60 mg/day for 103 days, improved fertilization rate in ICSI ( $P < 0.05$ ), although no significant improvement was observed in sperm parameters (Lewin and Lavon, 1997).

### In-vivo studies: no effect

Two different studies detected vitamin C to be of lesser value in improving pregnancy rate over the other medication given for treatment of infertility. A prospective randomized controlled study demonstrated that only 13% of patient population who were on medication with vitamin C were able to impregnate, while 17% pregnancy rate was seen in patients treated with clomiphene citrate (Abel *et al.*, 1982). Similar findings were reported with pregnancy rate of 19% in mesterolone treated patients compared with 18% in vitamin C treated patients (Hargreave *et al.*, 1984). It should be noted that vitamin C in both of these studies was compared with clomiphene citrate or mesterolone and not with placebo. The pregnancy rates of 13 and 18% reported in these studies may be due to a vitamin C effect.

## Discussion

Current evidence supports the use of a variety of systemic antioxidants for management of selected cases of male infertility as well as in-vitro media supplements during sperm preparation (**Table 1**). Different regimens using antioxidants were used in the treatment of infertile males according to their correlation with outcome parameters (**Table 2**). However, multiple in-vivo studies have been excluded for a variety of reasons, such as lack of comparison between pre- and post-antioxidant treatment results (Abel *et al.*, 1982; Hargreave *et al.*, 1984), study population was not infertile (Dawson *et al.*, 1992), use of uncharacterized substance (Niwa *et al.*, 1998), pilot studies/small study population (Lenzi *et al.*, 1992; Idriss *et al.*, 2000), and the route of antioxidant administration (Fahim *et al.*, 1985). It is abundantly clear that a definite conclusion cannot be easily drawn from the available studies on the effectiveness of antioxidants in male infertility due to multitude of factors.

The patient selection is one of the important aspects to be considered. OS is not the cause of male infertility in all patients. The rationale for antioxidant therapy in infertile patients should be based on raised OS status. A thorough workup should be carried out to identify known causes of OS and treatment should be directed towards them first. This includes correction of varicocele, and treatment of

**Table 2.** Summary of studies using oral antioxidants in the treatment of male infertility.

<i>Study and antioxidant</i>	<i>Antioxidant</i>	<i>Duration and dose</i>	<i>R/C/B</i>	<i>Sample size</i>	<i>Study population</i>	<i>Follow-up</i>	<i>Improvement in semen parameters</i>	<i>No improvement in semen parameters</i>
Lenzi <i>et al.</i> , 1993	Glutathione	2 months 600 mg/alternate day	Y/Y/Y	21	MFI (varicocoele and MAGI)	1 month	Motility, morphology	Concentration
Costa <i>et al.</i> , 1994	L-Carnitine	4 months, 3 g	N/N/N	100	Asthenozoospermia	2 months	Concentration, motility, morphology	
Lenzi <i>et al.</i> , 1994	Glutathione	2 months, 600 mg/alternate day	N/N/N	10	MFI (varicocoele, MAGI)	No	Concentration, motility, morphology	
Kessopoulou <i>et al.</i> , 1995	Vitamin E	3 months, 600 mg	Y/Y/Y	30	MFI patients with high ROS	4 months	Zona-binding assay (unfertilized human oocyte)	Concentration, motility, morphology, ROS
Moilanen and Hovatta, 1995	Vitamin E	3 weeks, 600 mg, 800 mg, 1200 mg	N/N/N	15	Infertility screening and volunteers	No		Concentration, motility, viability
Iwanier and Zachara, 1995	Selenium	3 months, 200 µg	N/N/N	33	Subfertile	No		Concentration, motility, morphology
Geva <i>et al.</i> , 1996	Vitamin E	3 months, 200 mg	N/N/N	15	ART patients	No	MDA concentration	Ultra morphology
Vežina <i>et al.</i> , 1996	Vitamin E, selenium	6 months, 400 mg vitamin E, 100 µg 1 month and 200 µg 5 months Se	N/Y/N	9	MFI (OAT)	2 months	Motility, morphology, viability	Concentration,
Suleiman <i>et al.</i> , 1996	Vitamin E	6 months, 300 mg	Y/Y/Y	110	Asthenozoospermia	No	Motility, MDA concentration	
Okada <i>et al.</i> , 1997	Pentoxifylline	300 mg 4 months then 1200 mg 4 months	N/N/N	33	Asthenozoospermia	No	Motility	ROS, zona-free hamster egg penetration test
Kodama <i>et al.</i> , 1997	Vitamins E, C, glutathione	2 months, 200 mg vitamin C, 200 mg vitamin E, 400 mg GSH	N/N/N	36	Infertile	No	Concentration, MDA concentration, DNA damage	Motility, morphology
Lewin and Lavon, 1997	Coenzyme Q <sub>10</sub>	103 days, 60 mg	N/N/N	17	MFI	No	Fertilization rate	Concentration, motility, morphology
Scott <i>et al.</i> , 1998	Selenium, vitamins E, C, A	3 months, 100 µg Se, or Se with 1 mg vitamin A, 10 mg vitamin C, 15 mg vitamin E	Y/Y/Y	64	46 OAT, 16 subfertile	1 month	Motility	Concentration

*continued overleaf...*

Table 2 continued

Rolf <i>et al.</i> , 1999	Vitamins C, E	56 days, 1000 mg vitamin C, 800 mg vitamin E	Y/Y/Y	33	Asthenozoospermia	2 months		Concentration, motility, morphology, viability
Comhaire <i>et al.</i> , 2000	Vitamins E, A, acetylcysteine	6 months, 600 mg acetylcysteine or 30 mg $\beta$ -carotene, 180 mg vitamin E	N/Y/N	27	Infertile	No	ROS, 8-OH-dG	Concentration, motility, morphology
Vicari and Calogero, 2001	Carnitine, acetyl-carnitine	3 months, 2 g carnitine, 1 g acetylcarnitine	N/Y/N	54	MI (PVE)	3 months	Motility, viability, WBC, ROS	Concentration, morphology
Vicari <i>et al.</i> , 2002	Carnitine, acetyl-carnitine	4 months, 2 g carnitine, 1 g acetylcarnitine	Y/Y/N	98	MFI (PVE)	3 months	Motility, viability, WBC, ROS	Concentration, morphology
Keskes-Ammar <i>et al.</i> , 2003	Vitamin E, selenium	3 months, 400 mg vitamin E, 225 $\mu$ g selenium	Y/Y/N	53	Infertile and volunteers	No	Motility, MDA concentration	
Lenzi <i>et al.</i> , 2003	L-Carnitine	6 months, 2 g/day	Y/Y/Y	100	MFI (OAT)	2 months	Concentration, motility	

R/C/B = randomized/controlled/blinding; Y = yes; N = no; ART = assisted reproduction techniques; MFI = male factor infertility; MAGI = male accessory gland infection; OAT = oligoasthenoatozoospermia; PVE = prostatic-vesiculo-epididymitis; ROS = reactive oxygen species; MDA = malondialdehyde; 8-OH-dG = 8-hydroxydeoxyguanosine.

inflammation and infection of the reproductive tract. In addition, patients should be advised to stop smoking, as it has been shown to be associated with OS.

Combination of antioxidants may be more useful in treatment of male infertility. The presence of several mechanisms to counteract OS in human reproductive system itself indicates that multiple antioxidants are required for defence against reactive oxygen species radicals, as individual antioxidant act through different mechanisms and on different free radicals. Single antioxidant supplementation can be justified if the concentrations of individual antioxidants are measured and only the deficient antioxidant is to be replaced.

The dosage of the antioxidant appears to be very critical. It is of importance to note that ROS are also required for physiological purposes. So, in principle, excessive antioxidants may cause impaired sperm function by inhibiting ROS. The dose range of antioxidants which does not suppress physiological concentrations of ROS and does not act as pro-oxidant should be determined by in-vitro experiments. This is further complicated by the fact that the minimum amount of ROS required for physiological function is as yet unknown. The formulation of the drug used also influences the effect. Formulation should be with the aim of reaching adequate seminal plasma concentrations of antioxidant to protect spermatozoa from OS in infertile patients. In addition, dosage along with duration of treatment is also vital.

One of the problems in assessing the efficacy of antioxidants in the treatment of infertility is the ability of spontaneous improvement of the sperm quality. It is also extremely difficult to recruit large numbers of subjects in long duration infertility studies. The main outcome measure which is relevant to the patient is pregnancy, which requires an extensive follow-up of the patient and also depends on female fertility status. The natural chances of a couple conceiving spontaneously should also be taken into consideration. Therefore, whether sperm quality improvement is because of natural fluctuations or due to the effect of the drug may not be clear.

In conclusion, the rationale and evidence supporting the use of antioxidants in infertile male patients with elevated OS do exist. The lack of a clear picture may be due to the insufficient, heterogeneous methods employed in the studies that were used for evaluation. A consensus is still required on type and dosage of antioxidants to be used and outcome parameters studied. To increase the sample size, multicentre trials should be planned.

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