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Glutathione as a treatment for male infertility

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The excessive generation of reactive oxygen species (ROS) by abnormal spermatozoa and by contaminating leukocytes has been identified as one of the few defined aetiologies for male infertility. As a consequence, work has begun on evaluating the role of antioxidants in the management of these patients. Glutathione plays a significant role in the antioxidant defences of the spermatogenic epithelium, the epididymis, and perhaps in ejaculated spermatozoa. The use of antioxidants *in vitro* appears to be of value in preserving fertilizing capacity, although no clinical data are available. Glutathione administered *in vivo* to patients who may have infertility secondary to excessive oxidative stress appears to act at the epididymis and during spermatogenesis, to improve the function of ejaculated spermatozoa. However, fertility studies have not yet been conducted. Controlled studies of glutathione and other antioxidants in patients with defined ROS pathology are urgently required.

Infertility probably affects at least one couple in six and the commonest single defined cause is sperm dysfunction (Hull *et al.*, 1985). Recent evidence has suggested that human semen quality is deteriorating by as much as 3% per year, leading to fears that male reproductive problems may be on the increase (Auger *et al.*, 1995). In spite of this there have, until recently, not been reliable ways to diagnose or treat patients suffering from this distressing problem, a situation which is a direct consequence of our lack of understanding of the causes of male infertility, and of the nature of the defects in sperm cell structure and biochemistry that underlie the loss in fertilizing potential.

In general, the medical treatment of the infertile male has been largely empirical and unrewarding, while surgical treatment has limited applicability, with the possible exception of varicocele ligation or embolization. In contrast, the past two years have seen remarkable achievements in the application of techniques of assisted reproduction to the treatment of couples with 'male factor' infertility. The pragmatic clinical application of the sophisticated techniques of micro-assisted fertilization has yielded clinically outstanding results. Unfortunately, its very cost and complexity is such that it cannot represent a final solution to the problem of the infertile male. The development of rational approaches to diagnosis and treatment must be based upon an understanding of the cellular pathologies that result in the production of defective spermatozoa. The recent characterization of a pathology comprising the excessive generation of reactive oxygen species (ROS), its association with defective sperm function, and the possible use of antioxidants in treatment will be the focus of this review.

Reactive oxygen species

Studies by Aitken and Clarkson (1987) and Alvarez *et al.* (1987) demonstrated that human spermatozoa can generate ROS, and it has also been shown that seminal leukocytes, particularly neutrophils, which contaminate most human sperm suspensions, can make a significant contribution to the ROS generation recorded (Aitken *et al.*, 1992a; Kessopoulou *et al.*, 1992). The clinical significance of this excessive ROS production was revealed by studies that have demonstrated that this pathology is associated with impaired sperm function, reflected in altered motility and sperm–oocyte fusion *in vitro* (Aitken and Clarkson, 1988; Aitken *et al.*, 1993a). In studies with infertile patients, increased ROS production was found to be negatively correlated with fertilization success in human IVF (Sukcharoen *et al.*, 1995) and with the achievement of pregnancy in prospective follow-up studies (Aitken *et al.*, 1991).

The mechanisms of ROS-induced damage to spermatozoa include an oxidative attack on the sperm plasma membrane lipids, leading to the initiation of a lipid peroxidation cascade, as a consequence of which the spermatozoa lose their capacity for movement, acrosome reaction and sperm–oocyte fusion (Jones *et al.*, 1979; Aitken *et al.*, 1993a). The membrane fusion events involved in fusion with the oolemma and in the acrosome reaction appear to be more vulnerable to ROS-induced damage than is overall motility (Aitken *et al.*, 1989). In addition, ROS may also affect the sperm axoneme, inhibit mitochondrial function, and affect the synthesis of DNA, RNA and proteins (de Lamirande and Gagnon, 1992a). The principal cytotoxic reactive oxygen intermediate involved in ROS-associated damage is probably hydrogen peroxide (H_2O_2) (de Lamirande and Gagnon, 1992b; Aitken *et al.*, 1993b), generated by the intracellular dismutation of superoxide anion ($O_2^{\cdot-}$) under the influence of superoxide dismutase (SOD). However, Alvarez and Storey (1989) suggested that the hydroxyl radical (HOO°), formed by the protonation of $O_2^{\cdot-}$, could be a potent indicator of peroxidative damage in human spermatozoa, although, in addition to H_2O_2, lipid peroxides generated as a consequence of the peroxidation process also appear to be profoundly cytotoxic, as do their degradation products (Selley *et al.*, 1991; De Lamirande and Gagnon, 1992b).

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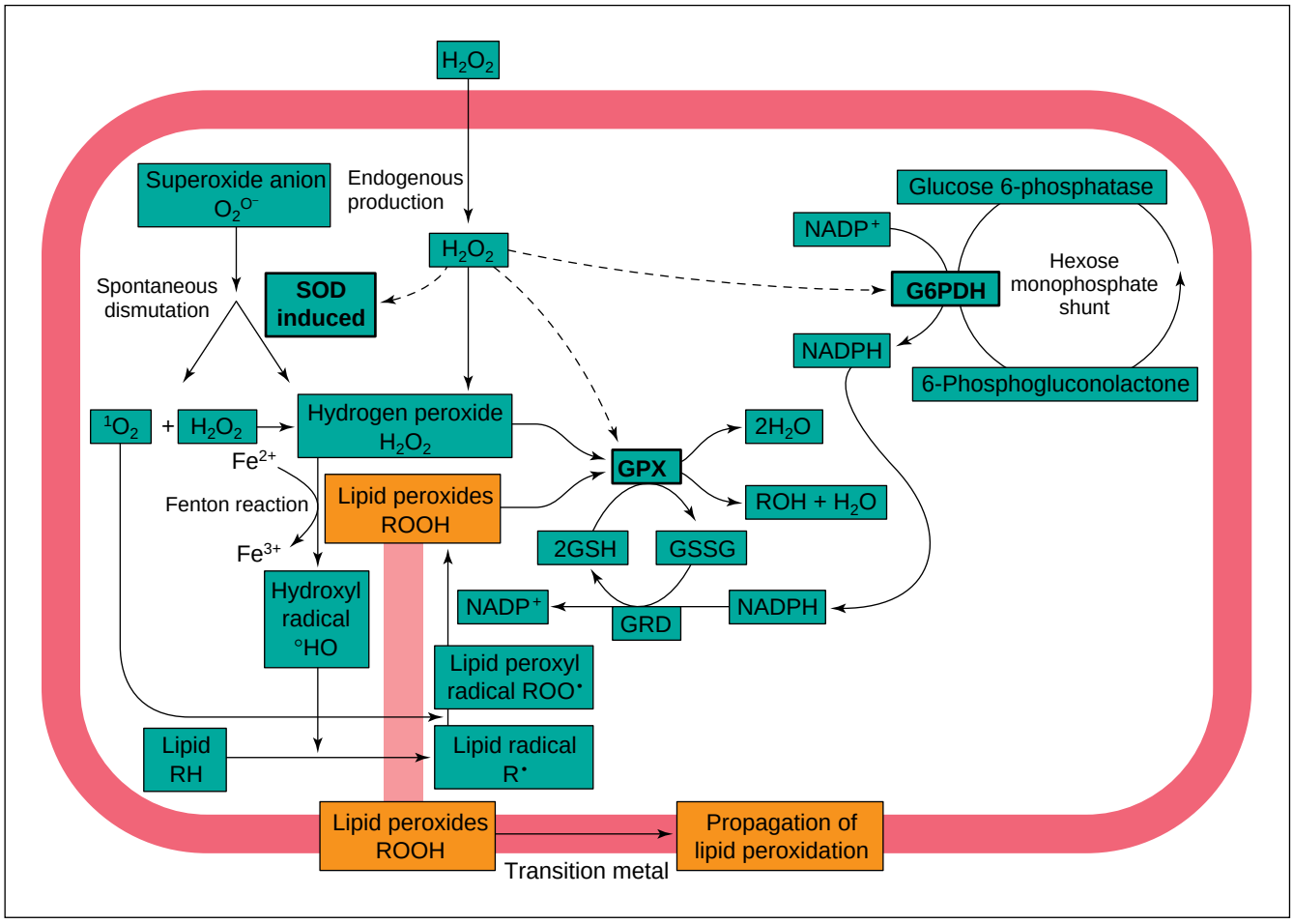

Fig. 1. Lipid peroxidation and antioxidant enzymatic defence systems in the spermatozoon. Hydrogen peroxide may decrease the activity of superoxide dismutase (SOD), glutathione peroxidase (GPX) and glucose-6-phosphate dehydrogenase (G6PDH) activities (- - - -), allowing the endogenous production of reactive oxygen species to result in the accumulation of toxic lipid peroxides and the development of lipid peroxidation (modified after Griveau *et al.*, 1995). GRD: glutathione reductase; GSSG: oxidized glutathione; GSH: reduced glutathione.

Lipid peroxidation

Human spermatozoa are known to be susceptible to loss of motility in the presence of exogenous H_2O_2, as a consequence of lipid peroxidation (Aitken *et al.*, 1993a). This susceptibility of human spermatozoa to oxidative stress is a consequence of the abundance of unsaturated fatty acids in the sperm plasma membrane, the presence of which gives this structure the fluidity it needs to engage in the membrane fusion events associated with fertilization. Unfortunately, the presence of double bonds in these molecules makes them vulnerable to free radical attack and the initiation of a lipid peroxidation cascade. Studies concerning the chemistry of lipid peroxidation in human spermatozoa (Aitken *et al.*, 1992b) imply that once this process has been initiated, its propagation is impeded, leading to the accumulation of lipid peroxides in the sperm plasma membrane. The extent to which lipid peroxidation occurs will depend upon the antioxidant strategies available to the spermatozoon. The loss of sperm function that results from lipid peroxidation reflects the negative impact that lipid peroxides have on membrane fluidity and the activity of key membrane-bound enzymes, such as Ca^{2+}/Mg^{2+}-ATPases, which are involved in maintaining calcium homeostasis within these cells.

Antioxidant defences

In common with all cell types, spermatozoa can defend themselves against oxidative damage. Although evidence regarding the existence and role of catalase in human spermatozoa is controversial (Jeulin *et al.*, 1989), they are known to possess two alternative defence mechanisms against the dioxygen species $O_2^{\cdot-}$ and H_2O_2, namely SOD and the glutathione peroxidase/reductase pair (GPX/GRD) (Alvarez *et al.*, 1987; Alvarez and Storey, 1989; Griveau *et al.*, 1995). The relative contribution of each of these defence mechanisms in normal men and in men with impaired spermatogenesis remains to be fully elucidated. Alvarez *et al.* (1987) suggested that SOD provides the major component of the antioxidant defences of human spermatozoa against the damaging effects of HOO°; however, Griveau *et al.* (1995) proposed a hypothesis that implicates SOD, glutathione peroxidase and glucose-6-phosphate dehydrogenase (G6PDH)

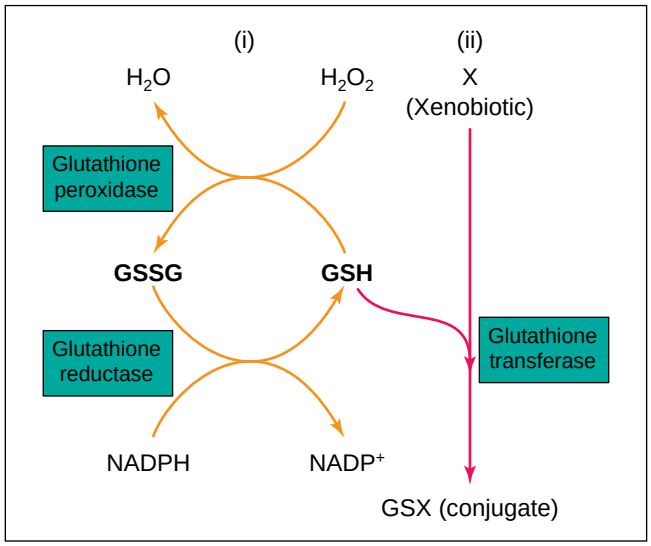

Fig. 2. (i) The glutathione redox cycle and (ii) the glutathione S-transferase reaction. GSH: reduced glutathione; GSSG: oxidized glutathione; GSX: conjugate between reduced glutathione and a xenobiotic.

in regulating the capacity of the cell to cope with damage induced by H_2O_2, associated with the ability of this oxidant to inactivate these enzymes (Fig. 1).

Whether antioxidant enzymes can ever make a profound contribution to the protection of ejaculated human spermatozoa is questionable, because these enzymes appear to be confined to the cytoplasm of the sperm midpiece. From this position, they would be unable to protect the plasma membrane covering the vulnerable regions of the sperm head and tail. Indeed, the relative lack of cytoplasmic space is a striking feature of human spermatozoa and may contribute to their vulnerability to oxidative stress, by reducing their capacity for antioxidant defence. In contrast, seminal plasma is a potent source of antioxidants, including SOD (Kobayashi *et al.*, 1991), uric acid, alpha-tocopherol and vitamin E (Jones *et al.*, 1979; Zini *et al.*, 1993). In addition, spermatozoa are known to be coated in lactoferrin, an iron-binding protein that plays a significant role in removing this important transition metal from sites where it may catalyse peroxidative damage. Seminal plasma is not the only extracellular fluid in the male reproductive tract to possess antioxidant factors. Recent reports of unique secreted forms of GPX and SOD in the mammalian epididymis emphasize the importance of epididymal plasma in protecting spermatozoa from peroxidative damage during their prolonged storage in the cauda epididymis (Perry *et al.*, 1992, 1993).

Thus, it is clear that ejaculated spermatozoa may be subject to oxidative stress, and there are intracellular and extracellular mechanisms to protect these cells from damage after spermiogenesis, during epididymal transit, and after ejaculation. However, oxidative stress may also arise during spermatogenesis, and be causally involved in the pathophysiology of male infertility. The antioxidant enzymes catalase, SOD, GPX and glutathione transferase (GTR) and the hexose monophosphate shunt (HMS) are present in whole rat testis, and significant changes in these enzymes are observed during testicular development. Sertoli cells express SOD and GTR, pachytene spermatocytes and round spermatids express SOD but not catalase, while GPX and GTR are found in both rat peritubular cells and Leydig tumour cells (Yoganathan *et al.*, 1989a, b). The ratio SOD : catalase plus GPX in the testis is high, possibly contributing to the vulnerability of the testis to oxidative stress (Peltola *et al.*, 1992). A consequence of a dependence of the testis on SOD as an antioxidant defence strategy is the vulnerability of testicular SOD to thermal inactivation. For example, experimental cryptorchidism, which raises testicular temperature, results in a marked impairment of testicular antioxidant defences, and a consequent rise in observed lipid peroxidation, possibly as a result of the greater susceptibility of testicular SOD to thermal inactivation (Ahotupa and Huhtaniemi, 1992). This raises the possibility that disorders that affect testicular temperature regulation, such as varicocele, may exert their effect through oxidative damage during spermatogenesis. It has even been suggested that environmental chemicals known to be testicular toxicants, including cigarette smoke, act through free-radical-dependent mechanisms (Peltola *et al.*, 1994).

Given this substantial body of evidence that oxidative stress is causally related to male reproductive dysfunction, attention has begun to focus on the possibility of using antioxidants, including glutathione, in the treatment of patients with male infertility.

Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) is the most abundant non-protein thiol in mammalian cells, being present in concentrations of 0.5 – 10 mmol l^{-1}. Cellular GSH plays a key role in many biological processes, including the synthesis of proteins and DNA and the transport of amino acids, but notably, it plays a key role in protecting cells against oxidation: the sulphydryl (SH) group is a strong nucleophile, and confers protection against damage by oxidants, electrophiles and free radicals (Meister and Anderson, 1983).

High concentrations of GSH have been observed in rat and mouse testis (Calvin and Turner, 1982; Grosshans and Calvin, 1985); a threefold increase in the concentration of GSH in rat testis was observed between days 8 and 29 of postnatal life, coinciding with the onset of spermatogenesis. Isolated spermatocytes and spermatids from hamsters contain large amounts of reduced GSH, and are capable of GSH synthesis, and of using GSH-dependent defence mechanisms (Den Boer *et al.*, 1989, 1990). Sertoli cells also actively synthesize GSH, and may play a role in its synthesis by germ cells. However, while substantial quantities of GSH are found in the testis, reproductive tract fluids, and epididymal spermatozoa, much less is present in ejaculated spermatozoa (Agrawal and Vanha-Perttula, 1988).

Glutathione is involved in a protective mechanism that involves inactivation of ROS, including peroxides formed in cellular oxygen metabolism. These toxic oxygen species may be detoxified via reduction by GPX, which is converted to oxidized glutathione (GSSG) in the process. In turn, oxidized GSH is reduced by GRD, in the presence of NADPH. In addition, other electrophilic foreign compounds (xenobiotics) may be detoxified in a reaction catalysed by a group of enzymes named glutathione S-transferases, by which they are conjugated with GSH (Den Boer *et al.*, 1990b) (Fig. 2).

Clinical uses of antioxidants: assisted conception

After ejaculation, spermatozoa are normally protected by the antioxidant environment of seminal plasma (Kobayashi *et al.*, 1991), and are rapidly separated from seminal leukocytes by migration through cervical mucus. In contrast, during the preparation of spermatozoa for use in assisted reproduction, the seminal plasma is removed, and spermatozoa and contaminating leukocytes are inadvertently brought into close proximity, in the presence of transition metals, and the absence of antioxidants (Aitken *et al.*, 1992a; Krausz *et al.*, 1994). Aitken and Clarkson (1988) showed that techniques of sperm preparation that involve a centrifugation step before the separation of motile cells are associated with a burst of ROS production from a subpopulation of cells, characterized by impaired motility and fertilizing ability. This burst of ROS has the effect of compromising the functional competence of normal spermatozoa in the same suspension. It has since been shown that leukocytic contamination of cell suspensions can also result in similar damage (Krausz *et al.*, 1992). As a consequence of these observations, the potential role of antioxidants in ameliorating such damage has begun to be examined, with studies involving vitamin E, GSH, catalase and SOD (Aitken and Clarkson, 1988; Griveau and Le Lannou, 1994). Vitamin E will suppress lipid peroxidation catalysed by ferrous ion *in vitro*, and consequently will rescue the capacity of ejaculated spermatozoa for sperm–oocyte fusion (Aitken and Clarkson, 1988). The use of other antioxidants, including SOD and GSH, was studied by Griveau and Le Lannou (1994), who examined the ability of SOD and GSH to influence the loss of motility and acrosome reaction rates in spermatozoa prepared by centrifugation. Both GSH and SOD have a protective effect on rates of acrosome reaction and loss of motility over 24 h, although only SOD preserves the capacity of the cells to exhibit hyperactivated motility. Baker *et al.* (in press) reported on the ability of various antioxidants to reduce the loss of motility caused by ROS generated by polymorphonuclear leukocytes. Glutathione, *N*-acetylcysteine, hypotaurine and catalase were all effective in preventing the impairment of sperm motility and average path velocity observed in the presence of activated polymorphonuclear leukocytes.

It is clear that the generation of ROS in suspensions of human spermatozoa prepared for assisted conception is of considerable clinical relevance. The need to devise strategies for minimizing contamination by leukocytes, and perhaps to extract any residual leukocytes was suggested by Aitken *et al.* (in press). The potential value of the addition of antioxidants, including GSH, to IVF culture systems is apparent, although, to date, this is not supported by any clinical studies.

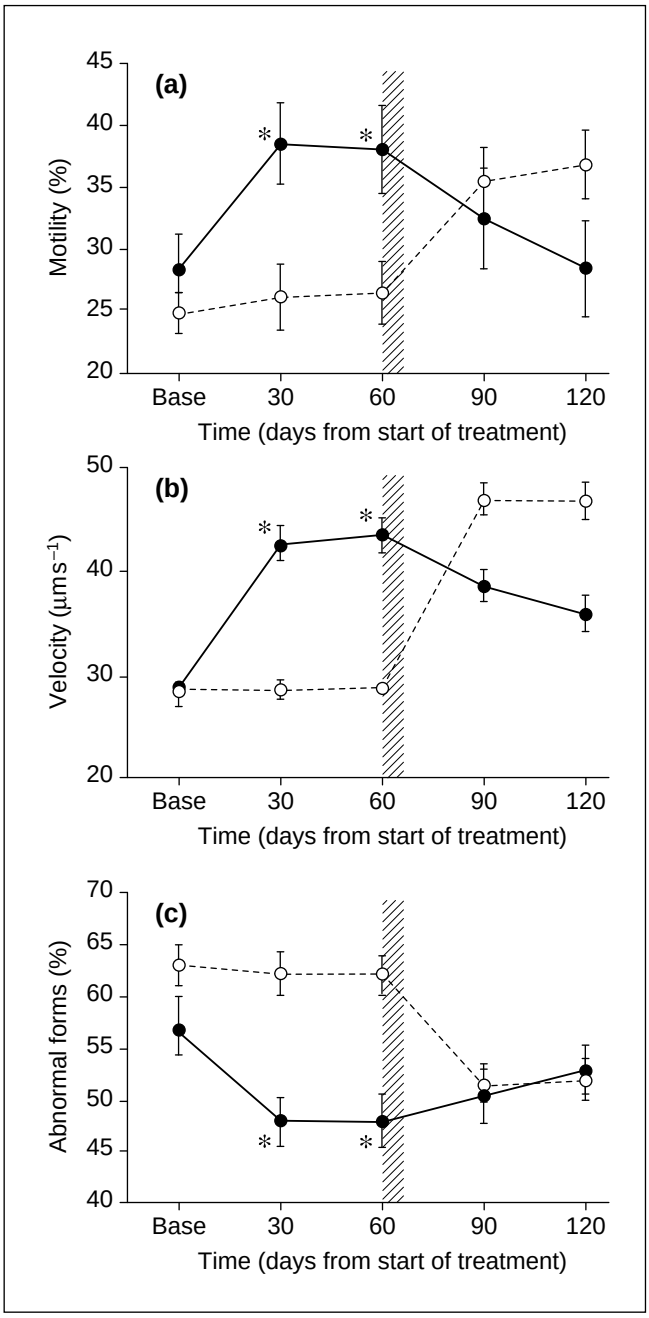

Fig. 3. Effect on overall sperm (a) motility, (b) velocity, and (c) morphology of treatment with glutathione (600 mg i.m. on alternate days) in men with varicocele and germ-free genital tract inflammation. Placebo-controlled crossover study, with crossover at 60 days (shading). Subjects starting on glutathione (GSH) (●) and crossing over to placebo; subjects starting on placebo (○) and crossing over to GSH. Data (from Lenzi *et al.*, 1993) are means ± SEM. *$P<0.05$.

Clinical uses of antioxidants: medical treatment of the infertile male

The administration of antioxidants to patients with 'male factor' infertility has begun to attract considerable interest. Unfortunately, there are no adequate randomized controlled trials on which to base any firm recommendations for clinical practice. Although there has been much interest in the administration of the readily available compound alpha-tocopherol (Vitamin E) (Moilanen *et al.*, 1993), there are no published data on the clinical use of this compound in male infertility associated with excessive ROS production. Glutathione, in contrast, is currently licensed as a pharmaceutical in only a few countries, for limited clinical indications, and has been shown to be of benefit in certain clinical situations (Dalhoff *et al.*, 1992; Tedeschi *et al.*, 1992; Holroyd *et al.*, 1993). Orally administered

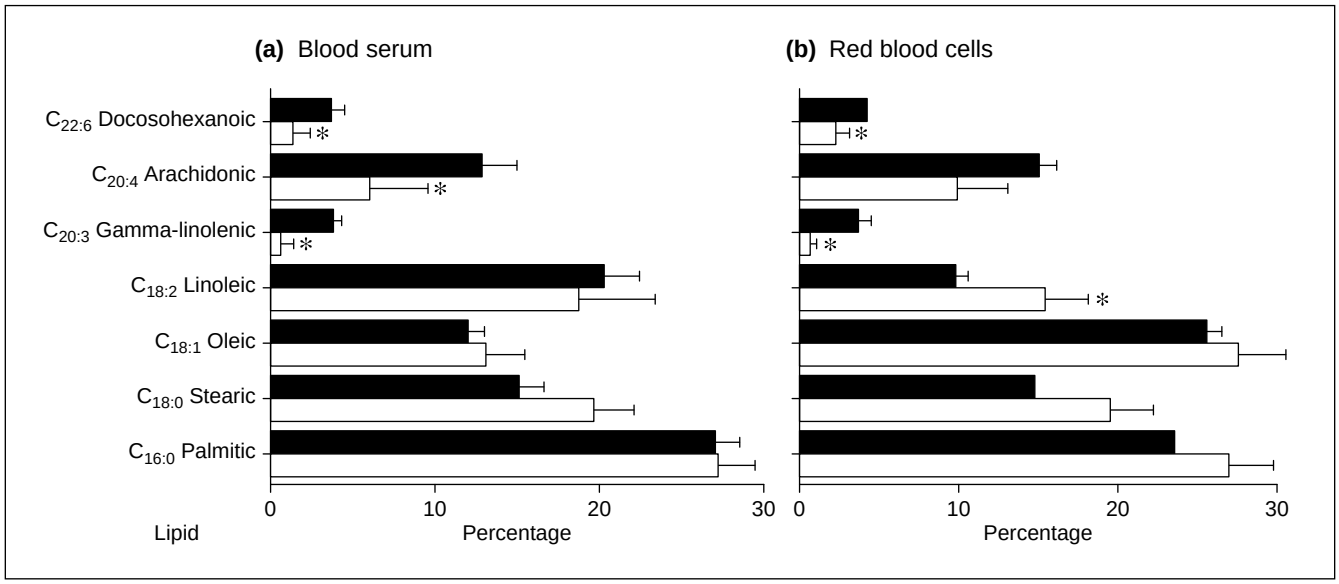

Fig. 4. Percentage of fatty acids in (a) blood serum phospholipids and (b) red blood cells in normospermic controls and infertile patients with varicocele or genital tract inflammation. Values are means ± SD. * Significantly different from control ($P \leq 0.001$). (■), control subjects; (□), infertile patients. Data from Lenzi *et al.* (1994)

GSH appears to be of limited value (Witschi *et al.*, 1992); hence, to be effective, the drug must be administered systemically, thus limiting its clinical application.

Lenzi *et al.* (1993) reported on a placebo-controlled crossover trial of 600 mg GSH, administered intramuscularly on alternate days, for a period of two months, to a group of 20 patients: ten with varicocele, and ten with 'germ-free genital tract inflammation'. Both of these pathologies might be expected to be associated with excessive ROS production, the former because of testicular thermal dysregulation, and the latter because of the presence of contaminating leukocytes. Unfortunately, the study design did not include any confirmatory assessment of the presence of excessive ROS generation before treatment. Nevertheless, when semen quality was assessed by classic WHO techniques (World Health Organization, 1992), and sperm movement was assessed with a computer-assisted image analysis system, parenteral GSH treatment resulted in significant improvements in overall motility, progressive motility, velocity, linearity, amplitude of lateral head displacement and beat cross frequency, together with a significant reduction in the proportion of forms with abnormal morphology (Fig. 3). Although the magnitude of the improvements was not large, the increases in velocity, for example, were apparent within 30 days of starting treatment, and persisted for some time after the cessation of treatment, suggesting effects both on epididymal spermatozoa and on the seminiferous epithelium.

The same group reported on serum and erythrocyte lipid profiles in a group of ten infertile patients before and after treatment with GSH, and in a group of 50 normospermic control subjects (Lenzi *et al.*, 1994). The infertile subjects were found to have a lower content of long chain polyunsaturated fatty acids, such as docosohexanoic ($C_{22:6}$ n3), arachidonic ($C_{20:4}$ n6), di-homo-γ-linolenic ($C_{20:3}$ n6) and linoleic ($C_{18:2}$ n6) acid (Fig. 4), and treatment with GSH resulted in significant increases in the observed content of di-homo-γ-linolenic acid and arachidonic acid after therapy for 60 days. This appeared to be associated with a fall in the lipid peroxidation potential of the spermatozoa of the treated infertile subjects, and an increase in sperm motility. Long chain polyunsaturated fatty acids are derived from the metabolism of the essential fatty acids linoleic acid ($C_{18:2}$ n6) and α-linoleic acid ($C_{18:3}$ n3) by elongation and desaturation in a hepatic metabolic pathway involving Δ-6-desaturase. It is not clear whether the lower content of polyunsaturated fatty acids observed in the infertile men reflects a systemic disorder of lipid metabolism, although the normal content of precursor linoleic acid and vitamin E would tend to suggest that altered dietary intakes are not responsible (Lenzi *et al.*, 1994). Others have suggested that diets rich in polyunsaturated fatty acids may result in alterations in sperm function (Diaz-Fontdevila and Bustos-Obregon, 1993). The relative deficiency of unsaturated fatty acids in the cell membranes of infertile patients could reflect the fact that the spermatozoa from these patients had been subjected to oxidative stress, although how parenterally administered GSH acts to prevent the consequent lipid peroxidation is less apparent. It is, for example, uncertain whether systemically administered GSH can gain access to the intracellular pool, although it has been shown that GSH, administered as an aerosol to patients with pulmonary GSH deficiency, becomes involved in the GSH cycle and appears as oxidized GSH in lung fluid (Holroyd *et al.*, 1993). The rapid onset of effects on motility suggest an epididymal site of action, in keeping with reports of secreted forms of GPX in mammalian epididymis and emphasizing the importance of epididymal plasma in protecting spermatozoa from peroxidative damage during storage in the cauda epididymis (Perry *et al.*, 1992, 1993). The sustained improvement of sperm movement after cessation of therapy suggests an effect on the seminiferous epithelium, in keeping with reports that round spermatids possess GSH-dependent defence mechanisms (Den Boer *et al.*, 1990a).

Conclusions

The description of excessive ROS production and the consequent process of lipid peroxidation leading to impaired sperm function represents the first biochemically defined cause of male reproductive dysfunction. Building on this, and on an understanding of the antioxidant defences of the testis, epididymis and spermatozoon, the use of antioxidants, notably GSH, in treating this problem has begun to be addressed. Initial results from studies *in vitro* and *in vivo* are promising, with clear evidence of improvements in critical aspects of sperm function after treatment. Unfortunately, studies undertaken to date are flawed by having failed to characterize clearly the cellular pathology present, and as yet, there are no data on the important clinical outcome – fertilization or pregnancy. Such studies are urgently needed.

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