Human Reproduction Update 1996, Vol. 2, No. 3 pp. 246–256

Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy

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This article is, in part, a review of present knowledge regarding the lipid metabolism of the sperm cell and the lipid composition of the sperm plasma membrane. It is also a summary of our research on this topic, reporting published and unpublished data. The article tries to cover both basic and clinical research. Sperm cells use lipid metabolic pathways for the production of part of their energy. The double leaflets of the membrane are not simply a passive, bilayer, lipidic film in which the receptors receive their molecular specific signals, but are a very specialized structure. Complete maturation of the lipids of the sperm cell membrane is reached after passage of the spermatozoon through the epididymis. A specific composition of phospholipids and a significant concentration of polyunsaturated fatty acids (PUFA) have been shown to be present in sperm membranes. Plasmalogen is a special kind of phospholipid found exclusively in spermatozoa and other cells with the capacity to react promptly to stimuli. In addition, we have found a high concentration of the *n*-3 PUFA family in the sperm membrane. Seminal plasma has a highly specialized scavenger system that defends the sperm membrane against lipoperoxidation. Various pathologies and systemic predisposition can lead to an antioxidant/pro-oxidant disequilibrium. Clinical trials with natural scavengers could be a useful research area in which to seek a treatment for these pathologies. Of the natural scavengers, glutathione has been shown to restore the physiological constitution of PUFA in the cell membrane under certain conditions.

Key words: glutathione/lipoperoxidation/PUFA/ROS/sperm membrane

Introduction

The sperm plasma membrane plays a very active role in sperm fertilization capacity and in spermatozoon–oocyte cross-talk. Its biochemical constitution is one of the main fields of interest in the study of sperm physiology and pathology.

Spermatozoa are polarized cells with structurally and functionally distinct domains. The two leaflets in the membrane of the cap region, overlying the acrosomal vesicle, constitute the only area sensitive to the capacitation stimuli. When the various steps of capacitation have induced an increase in the fluidity of the membrane in this region, a fusogenic process starts between this membrane and that of the outer acrosomal vesicle. The final event is the formation of pores that allow a dispersion of the acrosomal enzymes (acrosine and hyaluronidase). A further increase in the fusion process allows the formation of mixed pseudovesicles consisting of both the plasma and the outer acrosomal membrane. Towards the end of this membrane fusion, clearly shown in animal models but only partly confirmed in humans, spermatozoa can penetrate the zona pellucida.

Current theories of membrane fusion suggest that membrane fluidity is a prerequisite for normal cell functions and that the fluidity and flexibility of cell membranes are mainly dependent on their lipid constitution.

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Lipids of the sperm plasma membrane

The early analyses of sperm lipids, carried out during pioneer studies of mammalian and non-mammalian sperm biochemistry, showed the presence of neutral fatty acids, cholesterol, phospholipids (mainly lecithin, cephalin and sphingomyelin) and glycolipids (Koelliker, 1856; Miescher, 1878, 1897; Matthews, 1897; Sano, 1922). Subsequent analyses have shown that phospholipids are the most representative lipid fraction of the sperm cell membranes and, of these, phosphatidylcholine and phosphatidylethanolamine are the major components (Mann, 1964; Mann and Lutwak-Mann, 1981). A significant proportion of the phospholipid is in the form of plasmalogens, a quite distinct group of aldehydogenic phosphatidyl lipids that contain one fatty acid esterified with glycerol and an unsaturated ester with a long carbon chain (C-20, C-24). The hydrolysis of a plasmalogen yields a fatty aldehyde and a fatty acid. The exact role of these particular lipids is not completely known. However, it is interesting to note that a similar membrane lipid constitution has been described in cells of the nervous system (Mattew and Van Holde, 1990), i.e. other specialized cells devoted to signal transduction and cell-to-cell interaction. In this connection, it has been proposed that radical molecules may be considered as intracellular second messengers, transducing the signals from the outer to the inner leaflet of the membrane (Cornwell and Moriski, 1984).

The definitive lipid pattern of ejaculated spermatozoa is reached only after epididymal maturation. This was first demonstrated with ram testicular and ejaculated spermatozoa, which showed different lipid patterns (Scott *et al.*, 1967; Poulos *et al.*, 1975). Oxidative metabolism of endogenous fatty acids with up to two-carbon acyl-fragments could be particularly active in the epididymis and is probably correlated with the high concentration of carnitine and acetylcarnitine, which facilitate the passage of fatty acids within mitochondria, the site of β-oxidation (Mann and Lutwak-Mann, 1981).

A complete rearrangement of the membrane structure, both of proteins and lipids, has been shown in rams (Wolf *et al.*, 1986), guinea pigs (Myles and Primakoff, 1984; Cowan *et al.*, 1986) and rats (Hall *et al.*, 1981; Gaunt *et al.*, 1983) during sperm passage through the epididymis. It is also possible that biomembrane fluidity in human spermatozoa can increase, as the degree of unsaturation (associated with the fatty acyl component of phospholipids) increases when spermatozoa pass from the caput to the cauda of the epididymis. This would indicate an active sperm lipid metabolism. Furthermore, an asymmetrical transverse distribution of phospholipids has been demonstrated in ram sperm membrane, with aminophospholipids mainly located in the inner leaflet and choline-containing phospholipids in the outer leaflet. The presence of an aminophospholipid translocase in the membrane, together with the results of fluorescence studies, suggest a transbilayer movement of phospholipids and their transverse segregation in the fusion process during fertilization (Muller *et al.*, 1994).

Analyses of the fatty acid pattern of membrane phospholipids and plasmalogen of human spermatozoa have demonstrated significant levels of polyunsaturated acids. Three families of polyunsaturated fatty acids (PUFA) have been classified according to the distance of the first double bond to the methyl terminal, i.e. *n*-3, *n*-6 and *n*-9. α-Linolenic acid (C18:3 *n*-3), linoleic acid (C18:2 *n*-6) and oleic acid (C18:1 *n*-9) are the parent fatty acids of the different families. Long-chain PUFA in cell membrane phospholipids derive from the metabolism of the essential linoleic acid (C18:2 *n*-6) and α-linolenic acid (C18:3 *n*-3). These di-unsaturated fatty acids are normally present in the diet and are converted into their long-chain derivatives by a series of elongation and desaturation reactions (Table I), mainly in the liver. Δ^6-Desaturase, a rate-limiting enzyme, is probably regulated by the lipid composition of hepatic microsomes and by the presence of vitamin E and selenium, which could act as co-factors of the enzyme (Infante, 1986).

Table I. The metabolic pathways of *n*-6 and *n*-3 families of polyunsaturated fatty acids. The relevant enzymatic activities are listed between them. Linoleic acid (C18:2 *n*-6) and α-linolenic acid (C18:3 *n*-3) are considered 'essential fatty acids'

Linoleic acid		α-Linolenic acid
C18:2 *n*-6		C18:3 *n*-3
↓	Δ^6-desaturase	↓
γ-Linolenic acid		Octadecatetraenoic acid
C18:3 *n*-6		C18:4 *n*-3
↓	elongase	↓
Di-homo-γ-linolenic acid		Eicosatetraenoic acid
C20:3 *n*-6		C20:4 *n*-3
↓	Δ^5-desaturase	↓
Arachidonic acid		Eicosapentaenoic acid
C20:4 *n*-6		C20:5 *n*-3
↓	elongase	↓
Docosatetraenoic acid		Docosapentaenoic acid
C22:4 *n*-6		C22:5 *n*-3
↓	Δ^4-desaturase	↓
Docosapentaenoic acid		Docosaesaenoic acid
C22:5 *n*-6		C22:6 *n*-3

Table II. Fatty acid composition of sperm cell membranes. Each fatty acid class is given as a percentage of the total. The results represent the mean ± SD of five different samples from fertile subjects analysed in duplicate. The fatty acids were analysed by a combined gas chromatography–mass spectrometry technique as previously described (Lenzi *et al.*, 1994)

Source of cell membranes	Major polyunsaturated fatty acids							Other fatty acids
	C16:0	C18:0	C18:1	C18:2	C20:3	C20:4	C22:6	
Whole spermatozoa	31.5 ± 3.6	13.5 ± 2.1	18.5 ± 2.5	8.5 ± 2.2	0.21 ± 0.1	5.5 ± 1.2	22.5 ± 3.2	2.2 ± 0.5
Percoll-selected spermatozoa	25.6 ± 4	9.4 ± 2.2	10.5 ± 1.6	6.5 ± 1.2	0.32 ± 0.1	10.5 ± 1.5	34.5 ± 4.2	2.8 ± 0.4
Blood plasma[a]	26.8 ± 1.4	14.9 ± 1.7	12.1 ± 1.2	23.5 ± 2.3	3.9 ± 0.51	12.7 ± 1.8	3.8 ± 0.85	2.07 ± 0.2
Red blood cells[b]	23.6 ± 0.2	14.7 ± 0.36	25.5 ± 0.9	9.78 ± 0.9	1.37 ± 0.09	15.13 ± 0.95	4.1 ± 0.16	2.29

[a]From Passi *et al.* (1993).
[b]From Lenzi *et al.* (1994).

The activity of different lipid-dependent membrane-bound enzymes, including the second messenger systems, as well as membrane resistance to physical and chemical stress, depends on the fatty acid composition of cell membranes. In particular, PUFA are known to contribute to membrane fluidity and flexibility (Fleming and Yanagimachi, 1981; Meizel and Turner, 1983; Israelachvili *et al.*, 1990). Furthermore, PUFA are the precursors of prostaglandins and leukotrienes, important factors in both sperm motility and inflammatory processes. Prostaglandin E and 19-hydroxy-prostaglandin E have been shown to be related to sperm motility. Forward sperm motility is seen only when the concentrations of these substances lie within a relatively limited range of normality (Isidori *et al.*, 1980).

The fatty acid pattern of the membranes in human ejaculated spermatozoa has been studied by our group using combined gas chromatography–mass spectrometry (Lenzi *et al.*, 1994). Spermatozoa were analysed after washing (whole spermatozoa) and following sperm selection on Percoll gradients (Percoll-selected spermatozoa). The first method allows a simple separation of spermatozoa, leukocytes, germinal cells and other cells and debris from seminal plasma, whilst the second selects only morphologically normal sperm populations. In our study, this second method was used to eliminate all interference from cells other than spermatozoa. Preliminary data have shown significant differences in samples obtained by the two methods with regard to the percentage of PUFA. In whole spermatozoa the percentage of PUFA was 36–39%, whereas in Percoll-selected spermatozoa PUFA represented 48–52% of the total fatty acids. In particular, higher concentrations of C20:4 *n*-6 and C22:6 *n*-3 were recovered in Percoll-selected sperm cells, indicating that this pattern is characteristic of morphologically normal sperm cells. The PUFA pattern differences between whole and Percoll-selected spermatozoa suggest that the presence of contaminant cells other than normal spermatozoa modifies the percentage of each fatty acid. These results show that normal spermatozoa possess a higher percentage of the most representative PUFA (C22:6 *n*-3) than those detected in blood serum phospholipids and in other cell membranes such as erythrocytes (Table II). This suggests an active fatty acid metabolism and desaturation either during spermatogenesis or during epididymal sperm maturation. A high percentage of PUFA has already been noted in human and in mammalian spermatozoa (Poulos and White, 1973; Jones *et al.*, 1979), and significant differences in the percentage have been observed depending on the type of diet and, in animals, on the season (see Mann and Lutwak-Mann, 1981).

The degree of PUFA unsaturation in the membranes indicates that spermatozoa are extremely sensitive to external stimuli. The fertilizing function of spermatozoa could explain why a sperm cell has a fluid, flexible and very active membrane, which can easily be destabilized and activated. Similar percentages of PUFA, and especially of C22:6 *n*-3, are found in the membranes of nerve cells or cells derived from the neural crest, such as melanocytes (Vance *et al.*, 1985; Picardo *et al.*, 1990).

The essential role of PUFA in membrane constitution and in the fertilization process has been confirmed by experimental data on rats fed with an essential fatty acid-deficient diet. Together with decreased concentrations of PUFA in both red blood cells and serum, these animals showed a degeneration of the seminiferous tubules, a progressive decrease in germinal cells and an absence of spermatozoa in the lumina of the seminiferous tubules and epididymis (Leath *et al.*, 1983). These alterations and subsequent infertility are related to the marked reduction in the proportion of arachidonic acid in the total fatty acid content of the testis.

General mechanisms of lipoperoxidation and scavenger systems

An alteration in the lipid membrane constitution has been postulated as a common base for sperm pathology in many andrological diseases (e.g. varicocele, germ-free genital tract inflammation). In fact, PUFA and cholesterol are the main targets for free radical damage, and an inverse relationship between lipid peroxides and sperm motility has been clearly demonstrated (Aitken, 1991).

Various chemically dissimilar species can be considered to be free radicals, which are defined as molecules with an unpaired electron that react promptly with other free radicals or non-radicals (Pryor, 1984). In sperm pathology, particular attention has been devoted to oxyradicals. These include the superoxide anion radical (O_2^-), the hydroxyl radical (HO•) and nitrous oxide (NO). Sometimes, hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydrochlorous acid (HOCl) are also considered to be oxyradical species. These cannot be classified as free radicals because they still contain a pair of electrons in the outer orbital. Therefore the term reactive oxygen species (ROS) is preferentially used to cover all of these chemical species. Molecular oxygen is a diradical gas, possessing two unpaired free electrons that have parallel spin and the same quantum number (electron pairs usually have antiparallel spins), and therefore the single-electron reduction with the consequent generation of superoxide anion radicals is more frequent than the two-electron reduction.

In aerobic cells, the most important sources of O_2^- (half-life 0.4–1000 μs) are the electron transport chains of mitochondria and the endoplasmic reticulum. For every four electrons fed into a cytochrome oxidase complex, a molecule of O_2 is reduced to two molecules of water. Some components of the electron transport chain, in particular the NADH-coenzyme Q (CoQ) reductase complex and the reduced forms of CoQ, 'leak' a few electrons onto O_2, producing the univalent reduction of O_2 to give O_2^-, H_2O_2 and HO• (univalent pathway or univalent leak). The rate of the univalent pathway, and the consequent formation of ROS, is significantly increased by uncouplers of oxidative phosphorylation, hyperbaric O_2 treatment, and during pathological conditions such as ischaemia/reperfusion syndrome and ageing, and by alterations of mitochondrial lipids occurring when there is a deficiency of PUFA and lipoperoxidation processes. In fact, as an integral part of the membrane system, mitochondria contain a relatively high proportion of phospholipids with a high degree of unsaturation. Superoxide anion radicals are also generated in microsomes and plasma membranes under normal conditions (Foreman and Boveris, 1984). In the microenvironment of cell membranes, the formation of hydroperoxyl radicals from superoxide anion radicals is favoured. The hydroperoxyl radical, which is one of the more powerful oxidants, may be the predominant form of superoxide in phospholipid membranes. It is able to peroxidize PUFA and thus initiate a chain reaction (Bielsky *et al.*, 1983).

Hydrogen peroxide is the most stable intermediate of oxygen reduction. It is generated during the univalent reduction of oxygen and can be produced by different cells involved in the inflammatory processes, by a membrane NADH oxidase system and by the xanthine/xanthine oxidase system. In contrast to O_2^-, hydrogen peroxide crosses plasma membranes nearly as freely as water. It can be cytotoxic and is a weak oxidizing agent, but in the presence of transitional metals such as iron or copper it produces the more dangerous hydroxyl radicals by the Fenton reaction.

Hydroxyl radicals are generated during the respiratory burst of neutrophils and macrophages and they are the cytotoxic products of the less toxic superoxide anion radicals and hydrogen peroxide. Degradation of lipid hydroperoxides by reaction with superoxide anion radicals or the reaction of hydrogen peroxide with transition metals leads to the generation of hydroxyl radicals. The hydroxyl radical is extremely reactive (half-life ≈ 0.3 ns). It combines with any molecule at or very close to its site of formation with rate constants of 10^9–10^{10} M^{-1}/s^{-1}, so that almost everything present in a cell at high concentration can be considered a good HO^- scavenger. For this reason, the hydroxyl radical can cause biological damage if generated in close proximity to a potential target molecule essential for cell life or directly at a critical cellular target site.

Cellular homeostasis is generally regulated by the concentrations of peroxidizable substances, such as PUFA, and the effectiveness of the free radical scavenger systems. Therefore, oxidative stress can be defined as any disturbance in the balance between pro-oxidants and antioxidants in which the former prevail.

In mature spermatozoa, the high concentration of unsaturated lipids is associated with a relative paucity of oxyradical scavenger enzymes such as superoxide dismutase (SOD) catalase and glutathione peroxidase (Aitken, 1991). This relative deficiency is probably due to the virtual absence of cytoplasm in mature sperm cells. This, however, is compensated by the powerful antioxidant system in seminal plasma. Several studies have demonstrated that, in contrast to other biological fluids, seminal plasma contains significant levels of SOD, catalase and glutathione peroxidase together with significant concentrations of chemical antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E) and reduced glutathione (Dautner *et al.*, 1981). *In vitro*, seminal plasma significantly reduces the peroxidative

damage of iron-catalysed free radical generation. *In vivo*, a correlation between SOD in seminal fluid and sperm cell motility has been described (Mazzilli *et al.*, 1994).

Of the antioxidant systems, a special attribute of seminal plasma is the relatively high concentration of reduced glutathione. Glutathione, through its thiolic group, can react directly with hydrogen peroxide, superoxide anion and hydroxyl radicals, and its sulphydryl group can react with alkoxyl radicals and hydroperoxides, producing alcohols. Moreover, glutathione is the substrate of the selenium-containing enzyme glutathione peroxidase (the main enzyme involved in removing hydrogen peroxides) and of glutathione transferase (an enzyme which catalyses covalent reactions of glutathione with electrophilic substances such as quinones). In different biological systems, the glutathione redox cycle (involving the enzymes glutathione peroxidase and glutathione reductase) has an important role in protecting cells against oxidative damage (Giblin *et al.*, 1985; Inoue *et al.*, 1989; Meister, 1989).

The equilibrium equation involving reduced glutathione (GSH) and oxidized glutathione (GSSG) is

$$2GSH + H_2O_2 \xrightarrow{\text{glutathione peroxidase}} GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{glutathione reductase}} 2GSH + NADP^+$$

Generally, glutathione is present in millimolar concentrations in the cytosol, whereas its concentration is low in blood serum and in other biological fluids (Mann, 1964; Dautner *et al.*, 1981). Significant concentrations of this reducing agent have been correlated with the liquefaction process of seminal plasma and with the redox cycle of vitamin C. The oxidation of ascorbic acid to dehydroascorbic acid produces the generation of both ascorbyl radicals and hydrogen peroxide. Since the concentration of catalase in spermatozoa and seminal plasma is low, glutathione and glutathione peroxidase are the main agents that can remove the hydrogen peroxide generated (Inoue *et al.*, 1989).

The antioxidant system works in an integrated fashion. SOD dismutates the superoxide anion radical into hydrogen peroxide. There are two forms of SOD in mammalian cells: Cu-Zn SOD is found in cytosol, whereas Mn-SOD is localized in mitochondria. Ceruloplasmin can have similar scavenger effects. The hydrogen peroxide produced during the reactions has to be removed by the action of both catalase and glutathione peroxidase (Alvarez and Storey, 1989). Catalase is localized in peroxisomes, whilst glutathione peroxidase has been identified in the same subcellular organelles as SOD. If the function of these enzymes is insufficient to eliminate hydrogen peroxide completely, the Fenton reaction takes place in the presence of transitional metals. This reaction results in the production of toxic hydroxyl radicals. Vitamin E is a chain-breaking antioxidant and its role is to terminate 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 glutathione, producing thiyl radicals and oxidized glutathione. This last step can then be reversed by glutathione reductase. Consequently, 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.

Lipoperoxidation and sperm pathology

The harmful impact of oxyradicals and toxic compounds on sperm function has been well studied, with particular emphasis on the negative effects of ROS (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987; Aitken *et al.*, 1989; D'Agata *et al.*, 1990). The alteration of the fatty acid pattern of the membrane produces significant modifications (in most mammalian cells), including changes in the activity of various lipid-dependent enzymes and in the resistance to physical or chemical stress (Merril, 1989).

The risk to spermatozoa of lipid peroxidation can be assessed using a spectrophotometric assay to test the generation of malonyl-dialdehyde promoted by ferrous and ascorbate irons in the presence of thiobarbituric acid (Barber and Bernheim, 1967; Alvarez and Storey, 1982; Aitken *et al.*, 1989). Thiobarbituric acid evaluates the ferrous iron-catalysed breakdown of the pre-existing lipid hydroperoxides in the sperm plasma membrane and the subsequent propagation of a lipid peroxidation chain reaction through the generation of peroxyl and alkoxyl radicals (Aitken *et al.*, 1993).

ROS can be toxic for spermatozoa and can significantly affect sperm motility (Alvarez and Storey, 1982, 1989). The sources of ROS in the genital tract are the spermatozoa themselves, leukocytes during inflammatory processes, and ischaemia/hypoxia produced during vascular diseases. The principal ROS produced by spermatozoa seem to be the superoxide anion radical, which generates hydrogen peroxide spontaneously or following the activity of SOD (Alvarez *et al.*, 1987). Of the ROS produced, hydrogen peroxide is considered the most toxic for human spermatozoa (Aitken and Clarkson, 1987; Alvarez and Storey, 1989) since catalase but not SOD can inhibit the in-vitro cytotoxicity produced by the xanthine/xanthine oxidase system. Hydrogen peroxide may be toxic *per se*, even though its effect is likely to be indirect due to its weak oxidant

properties. It may act through the generation of hydroxyl radicals via metal-assisted Fenton reactions.

The generation of ROS has been correlated with sperm pathologies. In fact, ROS are readily detectable in a large percentage of infertile patients, while they are found at very low concentrations in fertile subjects. Experiments have shown that cells isolated from the 40–65% Percoll gradients are the main source of ROS and consist mostly of leukocytes and damaged spermatozoa. However, cells isolated at the 65–95% interfaces should be morphologically normal spermatozoa. In infertile patients, however, they too can produce ROS (Zini *et al.*, 1993; Zalata *et al.*, 1995). These results are in line with our findings, reported above, on the percentage of highly unsaturated PUFA found in Percoll-selected morphologically normal spermatozoa compared with whole spermatozoa. This supports the hypothesis that ROS production in patients suffering from sperm hypomotility is more likely to be the cause rather than the effect of decreased motility (Zalata *et al.*, 1995).

Particular attention has been given to the negative effects *in vitro* of ROS on spermatozoa and the implications of this in semen preparation for in-vitro fertilization programmes. However, some reports have shown that, *in vitro*, ROS can trigger physiological sperm functions, such as capacitation and hyperactivated motility (De Lamirante and Gagnon, 1993; Griveau and Le Lannou, 1994; Griveau *et al.*, 1994), and significant ROS production by spermatozoa has recently been shown during the capacitation process (De Lamirante and Gagnon, 1995). These positive effects are strictly related to the equilibrium between ROS and the scavenger systems. In assisted reproduction, great differences have been observed in the results of sperm selection techniques if the semen characteristics are not taken into account and if the sperm preparation methods are not chosen case by case (Mortimer, 1991). The culture medium for sperm preparation and the substances added to it must also be selected bearing the above in mind. For example, pentoxifylline, which has been very well studied, has a stimulating effect *in vitro* on iron-induced lipid peroxidation, which generally acts positively on membrane fluidity and physiological destabilization. However, it can also induce a destructive peroxidation chain reaction when the spermatozoa are more fragile than usual or when incubation is too long (Gavella and Lipovac, 1994).

Many andrological pathologies, including varicocele, infections and germ-free genital tract inflammation, have been associated with an increase in the risk of lipid peroxidation (Lenzi *et al.*, 1994). The same pathologies induce many biological and clinical effects, such as modifications in microcirculation, venous stasis and subsequent hypoxia, leukocyte activation and cell necrosis, all of which increase ROS in the semen. In fact, studies have shown ROS to be higher in the male partner of infertile couples suffering from selected andrological conditions (Aitken *et al.*, 1989; D'Agata *et al.*, 1990; Mazzilli *et al.*, 1994). Repeated centrifugation of spermatozoa can increase the generation of ROS, possibly due to the mechanical activation of cell membrane oxidative systems in addition to contact with damaged spermatozoa and leukocytes (Mortimer, 1991).

Based on the above, researchers developed the idea of using 'scavenger therapy' to treat some forms of dyspermia. Given the fact that it has usually proved impossible to identify the true aetiology of dyspermia, many non-hormonal therapies have been used to act directly on spermatozoa in the hope of improving their quality. This is understandable given the difficulty of making a diagnosis in this field of andrology. Treatments have varied over the years, but have tended to involve the use of carnitine, phosphatidylcholine, kallikrein, pentoxifylline and vitamins A, E and C (Mann and Lutwak-Man, 1981; Lanzafame *et al.*, 1994). Unfortunately, the real difficulty may be that andrologists only see infertile patients with dyspermia many years after the underlying pathology has already triggered sperm damage. It is obviously simpler and more practical in these circumstances to try to improve seminal parameters by prescribing drugs that act directly on gamete production or epididymal maturation. Controversial data have often resulted from the frequently uncontrolled studies carried out to support such therapies.

As previously mentioned, spermatozoa and seminal plasma have many scavenging systems to defend against the harmful effects of ROS and other toxic compounds. SOD and glutathione peroxidase are the most representative enzymes. Catalase activity has also been demonstrated. The main non-enzymatic scavenging systems are proteins (mainly albumin), glutathione, vitamins E and C, taurine and hypotaurine, and mercapturic acids (Halliwell and Gutteridge, 1989). Of these, glutathione seems to be most frequently used pharmaceutically, owing to its demonstrated antitoxic and antioxidant action in other degenerative pathologies (cirrhosis, neoplasiae, consequences of antiblastic therapies). Sulphydryl constituents of semen (cysteine, glutathione and ergothioneine) have been shown to play an important role in maintaining sperm motility and metabolism under experimental conditions (Mann and Lutwak-Mann, 1981) and, in isolated rat spermatids, glutathione can prevent the damage resulting from exposure to peroxidizing agents (Den Boer *et al.*, 1990). The pharmacology of exogenous administration of glutathione has yet to be completely studied and it is unlikely that the polar molecule can cross cell plasma membranes. However, it has been shown in experimental animals that glutathione

administration reduces stress-induced gastric injury without any modification of the tissue concentration of the antioxidant (Inoue *et al.*, 1989). One of the most important points in selecting glutathione as a therapy is its physiologically significant presence in seminal plasma. Even though it cannot cross cell membranes, this antioxidant can increase its concentration in biological fluids after systemic administration. It is able to reach the seminal plasma and concentrate there, thus exerting its physiological and therapeutic role (Lenzi *et al.*, 1993).

For all these reasons, our group has developed a research line related to the use of glutathione in sperm pathology and to its mechanism of action, which are briefly summarized below.

Firstly, we carried out a 2-month pilot study on the administration of glutathione (600 mg/day injected i.m.) to a group of patients with dyspermia associated with various selected andrological pathologies. Before treatment and also after 30 and 60 days of therapy we carried out standard semen analysis (World Health Organization Standard Procedure, 1992) and, to obtain objective data on sperm movements, we also carried out a computer analysis of sperm motility (Cell Soft, Cryo Resources). Glutathione therapy had a statistically significant effect on sperm motility patterns and sperm morphology. In particular, a significant difference between baseline and 60-day analyses was observed in the percentage of spermatozoa with forward motility and in the parameters of the sperm motility computer analysis. A significant reduction in the percentage of atypical forms was also seen. Sperm motility improved, especially in patients with chronic inflammation of the genital tract and in patients with varicocele (Lenzi *et al.*, 1992).

Secondly, we carried out 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 randomly and blindly assigned to treatment with one i.m. injection every other day of either glutathione 600 mg or an equal volume of a placebo preparation (for a detailed description of selection criteria and the cross-over statistical analysis results, see Lenzi *et al.*, 1993). 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. This effect must be due to a post-spermatocyte action, as the period of therapy was specifically chosen to be shorter than a complete spermatogenetic cycle and because early positive results were seen after the first month of treatment. These effects on sperm motility and morphology lasted beyond the therapy period. All this suggests that glutathione, even though it is unable to cross cell membranes, acts indirectly by improving the metabolic condition of the testicular–epididymal environment. The slight improvement observed in sperm concentration could be explained by a reduction in sperm phagocytosis at a post-testicular level (Lenzi *et al.*, 1993). These results were confirmed by another group using orally administered vitamin E and the same experimental design (placebo-controlled double-blind cross-over trial) (Kessopoulos *et al.*, 1995).

Finally, the use of glutathione (and SOD) *in vitro* has also been proposed for cases with oligozoospermia. This was to minimize possible sperm damage induced by the contact of healthy spermatozoa with pathological components of the semen during in-vitro manipulation for assisted reproduction (Griveau and Le Lannou, 1994). To study the in-vitro action of glutathione on human spermatozoa, we selected semen samples from semen-bank donors and infertile patients, with and without leukospermia. This last group was selected on the basis of postulated sperm damage caused by leukocyte-produced ROS (Aitken and West, 1990; Mortimer, 1991). We used microscopic semen analysis and computerized sperm motion analysis to determine the sperm parameters in basal semen and in post-rise spermatozoa obtained by layer and pellet-swim up in Tyrode's solution alone or containing 1 mg/ml of glutathione. In cases of leukospermia, we studied the effect of glutathione *in vitro* on basal semen, diluted semen (1:1 semen and Tyrode's solution + glutathione) and post-rise spermatozoa after layer and pellet-swim up. There were no significant differences between the sperm parameters of basal semen and semen diluted with Tyrode's solution + glutathione, nor were significant differences detected between layer swim up with and without glutathione in the medium. The only statistically significant difference was in sperm forward motility in leukospermic samples treated with pellet-swim up. In these samples, an increase was seen when the migration medium contained glutathione (Gandini *et al.*, 1993). These results indicate that glutathione protects sperm motility, *in vitro*, during pelleting, when there can be contact between seminal ROS, produced by leukocytes or damaged spermatozoa, and normal spermatozoa.

PUFA as markers of sperm pathology

As previously stated, oxidative stress may be defined as any imbalance between pro-oxidants and antioxidants in which the former prevail and produce a free radical cascade leading to a lipoperoxidative process. PUFA of phospholipids play a major role in membrane constitution and function and are one of the main targets of the lipoperoxidative process. Their degree of unsaturation is therefore an essential parameter in the ability of spermatozoa to maintain equilibrium in an oxidative environment. To understand the

therapeutic action of glutathione, we strictly selected infertile patients with unilateral varicocele and germ-free genital tract inflammation and studied the modifications produced by the mode of glutathione therapy on (i) semen variables and sperm membrane risk of lipoperoxidation (evaluated by thiobarbituric acid assay) and (ii) the pattern of fatty acids of phospholipids from blood serum and red blood cell membranes. Since it is practically impossible to evaluate the fatty acid pattern of sperm membrane phospholipids in patients with severe oligozoospermia, and red blood cells are considered to be a representative model of the constitution of cell membranes in general, they were used as an alternative.

An improvement in sperm concentration, motility, morphology and kinetic variables was also observed in this series of patients receiving glutathione therapy. These improvements were associated with a decrease in the concentrations of lipoperoxides in the spermatozoa and with an increase of the red blood cell concentrations of PUFA of phospholipids (Table III and Table IV). The arachidonic acid/linoleic acid ratio (a valuable indicator of the PUFA metabolism) in phospholipids of both red blood cells and spermatozoa before therapy was lower in the infertile patients than in fertile subjects. After therapy, together with the reduction of sperm lipid peroxidation, concentrations of the precursor di-omo-γ-linolenic acid and of arachidonic acid increased significantly in phospholipids of both red blood cells and serum (for more detailed data, see Lenzi *et al.*, 1994). However, their values remained lower than those of normal subjects. This suggested that, at least in part, the therapeutic action of glutathione is due to its general protective effect on the lipid components of the cell membrane. It is likely that glutathione also acted as a free radical scavenger in the epididymis, thus reducing the impact of the lipoperoxidative process generated by vascular or inflammatory pathologies. The significant reduction in the concentration of lipoperoxide in seminal plasma as detected by the thiobarbituric acid test supports this hypothesis. Moreover, the increase in the sperm cell concentration after 30 days of therapy (without variation in semen volume) indicates a reduction in sperm damage and subsequent epididymal reabsorption rather than a variation in spermatogenesis (Lenzi *et al.*, 1994). This appears to confirm experimental data indicating that rat spermatids utilize glutathione-dependent mechanisms against oxidative stress (Den Boer *et al.*, 1990). Our study has not completely defined the way in which glutathione improves the PUFA pattern of red blood cells. However, as fatty acid desaturation is regulated by the presence of selenium, the prosthetic group of glutathione peroxidase (Infante, 1986), we can postulate that the administered glutathione triggers the same enzymatic activities. Furthermore, from the above results, it is likely that the patients studied had an impairment of the desaturase enzymes, possibly due to genetic factors or unknown acquired pathologies leading to chronic systemic damage of all cell membranes. This constitutional alteration in cell membranes may increase the harmful effect of the oxyradicals generated in the epididymis following vascular or inflammatory processes, facilitating dyspermia. It seems likely that patients with systemic lipid membrane disturbances associated with andrological pathologies (e.g. varicocele and inflammation) express this membrane damage in spermatozoa and are prone to develop dyspermia. In this context, glutathione could act by modifying the biochemical constitution of the lipid membrane. This could confirm experimental data on rats fed a poor dietary regimen that show a deficiency of PUFA in serum and semen (Leath *et al.*, 1983; Brenner, 1984).

Table III. Lipoperoxides in patients before (T0) and at the end (after 60 days, T60) of glutathione therapy. Values are expressed in nmol malonyl-dialdehyde/10^8 spermatozoa

	T0	T60
Mean	14.98	11.84
SD	1.07	0.71
SE	0.48	0.32

t-test = 9.358; $P \leq 0.001$.

Table IV. Percentage of linoleic acid (LIN), arachidonic acid (ARA) and arachidonic/linoleic acid ratio (ARA/LIN) in blood serum (BS) and red blood cells (RBC) phospholipids of patients before (T0) and after 30 (T30) and 60 days (T60) of glutathione therapy. From Lenzi *et al.* (1994)

	Duration of glutathione therapy (days)			Significance of
	T0	T30	T60	T0 versus T60
LIN in BS	18.70 ± 4.40	18.25 ± 4.55	18.42 ± 7.23	$P < 0.05$
ARA in BS	6.07 ± 3.50	6.45 ± 2.67	8.61 ± 4.06	$P < 0.05$
ARA/LIN in BS	0.34 ± 0.13	0.32 ± 0.11	0.49 ± 0.21	$P < 0.05$
LIN in RBC	15.38 ± 5.25	15.99 ± 5.05	15.10 ± 3.61	$P < 0.05$
ARA in RBC	9.89 ± 3.08	10.00 ±4.59	17.56 ± 5.60	$P < 0.001$
ARA/LIN in RBC	0.61 ± 0.30	0.65 ± 0.24	1.19 ± 0.43	$P < 0.01$

To confirm this hypothesis, we selected two groups of patients suffering from unilateral varicocele, the first with normozoospermia and the second with dyspermia, and a control group of healthy fertile subjects. Preliminary results (unpublished data) did not show a significant difference between the PUFA of red blood cell membranes of the control group and the normozoospermic patients with a varicocele. However, there was a significant decrease of PUFA unsaturation in the majority of dyspermic patients. A follow-up study is obviously necessary to establish if congenital or acquired alterations in the PUFA of all cell membranes are related to varicocele-induced dyspermia. On the basis of only a transverse study, it is impossible to state whether there is a pathogenetic relationship between male gamete damage due to a lower resistance to oxyradical aggression against the sperm plasma membrane and vascular pathology of the testicular–epididymal region. It is striking that normozoospermic varicocele patients have PUFA in all cell membranes that are similar to those of control subjects while, to the contrary, dyspermic varicocele patients show a significant deterioration in this parameter. If these data are confirmed with a long-term follow-up study, it will become possible to establish a predictive index of the risk of varicocele-induced dyspermia; surgery could then be recommended only to young patients with PUFA alterations.

Conclusions

This article on the PUFA composition of the sperm cell membrane and on their physiology and involvement in pathological conditions has tried to highlight the fundamental role of these substances in the normal homeostasis of the male gamete.

As previously mentioned, some particular characteristics of the lipid constitution of the sperm membrane have been identified. The presence of plasmalogen and the high percentage of PUFA, mainly of the *n*-3 families, seem to be the most important findings. Sperm cells possess an active lipid metabolism, and the significant concentration of free fatty acids in the seminal plasma together with the concentration of carnitine clearly indicate that lipids are important sources of energy in the metabolism of sperm cells. Evaluating the overall biochemical composition of seminal plasma, which has higher concentrations of antioxidants than other biological fluids and blood serum, it seems likely that seminal plasma acts as a nutritive-protective medium for sperm cells. It can become a hostile medium when pathologies alter its anti/pro-oxidant equilibrium or when the sperm membrane is more fragile in natural conditions or as a result of in-vitro manipulation. A marker of this increase in fragility could be a disequilibrium in sperm membrane PUFA composition. Natural scavengers such as glutathione could represent a pathogenetic therapy in selected cases of ROS-associated sperm damage and subsequent male infertility.

Several points need to be clarified to define better the relationship between lipids, the PUFA composition of the plasma membrane and sperm cell functions. Firstly, it is important to determine whether sperm cells are capable themselves of desaturating essential fatty acids. Secondly, it would be extremely useful to demonstrate whether the high percentage of *n*-3 PUFA in the sperm membrane is necessary for normal fertilizing capacity. In addition, the correlation between systemic lipid metabolism and sperm cell functions should be further investigated. Finally, the physiological mechanism of action of the complex and of the single components involved in the antioxidant system in seminal fluid must be completely understood.

Acknowledgements

This work was supported by grants from the National Research Council (CNR) Target project – Prevention and Control of Disease Factors – Subproject 5 (Grant no. 95–00818-PF41) and the University of Rome “La Sapienza” – Faculty projects (40%–60%).

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Received on November 27, 1995; accepted on March 18, 1996