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LIPOPEROXIDATION DAMAGE OF SPERMATOZOA POLYUNSATURATED FATTY ACIDS (PUFA): SCAVENGER MECHANISMS AND POSSIBLE SCAVENGER THERAPIES

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1. ABSTRACT

The lipid metabolism in sperm cells is important both as one of the main sources for energy production and for cell structure. The double leaflets of the membrane should be considered not simply as a passive lipid film, but as a very specialized structure. The complete maturation of the sperm cell membrane is attained after testicular lipid biosynthetic processes and after passage through the epididymis. A special composition of membrane phospholipids, rich in polyunsaturated fatty acids (PUFA), and the different composition of sperm and immature germ cell membrane are described and discussed.

Testis germ cells as well as epididymal maturing spermatozoa are endowed with enzymatic and non-enzymatic scavenger systems to prevent lipoperoxidative damage. Catalase, superoxide dismutase and GSH-dependent oxidoreductases are present in variable amounts in the different developmental stages. Phospholipid hydroperoxide GSH peroxidase (PHGPx) activity and alpha tochopherol of epididymal spermatozoa are considered in detail. Their distribution and roles in caput and cauda epididymal sperm cells are discussed.

Seminal plasma also has a highly specialized scavenger system that defends the sperm membrane against lipoperoxidation and the degree of PUFA insaturation acts to achieve the same goal. Systemic predisposition and a number of pathologies can lead to an anti-oxidant/pro-oxidant disequilibrium. Scavengers, such as GSH, can be used to treat these cases as they can restore the physiological constitution of PUFA in the cell membrane. The results of GSH therapy are presented and discussed.

2. PHOSPHOLIPIDS OF THE SPERM PLASMA MEMBRANE: THEIR PHYSIOLOGY AND ROLE

2.1. Preliminary remarks

Sperm membranes play a very active role in sperm fertilization capacity and in sperm-oocyte cross talk, and its biochemical constitution is one of the main field 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 are the domains sensitive to the capacitation stimuli (1). When the various steps of capacitation have induced an increase in the fluidity of the cap region, a fusogenic process, which possesses already the structural premise, starts between this membrane and the outer acrosomal vesicle membrane. The final event consists in 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. In order to better elucidate this topic, early analyses of sperm lipids were carried out by pioneer studies of semen biochemistry that showed, in mammalian and non mammalian spermatozoa, the presence of neutral fatty acids, cholesterol, phospholipids (mainly lecithin, cephalin and sphingomielin) and glycolipids (2-6). Authors also showed that the oxidative metabolism of endogenous fatty acids of up to two-carbon acyl-fragments could be particularly active in the epididymis. This is due to the high concentration of carnitine and acetylcarnitine that facilitate the passage of fatty acids into mitochondria, the site of beta-oxidation. A great attention has been dedicated in recent years, in the field of the lipids, to the role of cholesterol in the fusogenic events leading to capacitation. Cholesterol can reduce the insertion of proteins into plasma membrane phospholipids (7). Moreover, it has been demonstrated that cholesterol can inhibit the lateral motility of proteic receptors, modulating their activity and changing their conformation (8). This is also supported by the fact that the removal of cholesterol triggers membrane destabilization and allows protein migration, which is the basis of capacitation and the acrosome reaction (1). During the in vitro capacitation process, cholesterol concentration is reduced mainly in the plasma membrane of the acrosomal cap (1, 8). Albumin, which is one of the most widely investigated sterol acceptors that can promote efflux of cholesterol from the sperm plasma membrane, is considered to be one of the majors capacitating substances (9). The subsequent increase in membrane fluidity allows the migration of the receptors to the equatorial region. This migration or shedding of glyco-lipo-proteic components of the acrosomal membrane has been clearly shown in guinea pigs (10, 11). This has also been proposed recently for human spermatozoa using antisperm antibodies as probes of the membrane antigenic change during capacitation events. (12, 13). The last step is the triggering of the molecular signals that activate acrosome reactions, which involves many second messenger pathways. Furthermore, analysis of the lipid composition of sperm cell membranes has shown that phospholipids are the most representative lipid fraction and, of these, phosphatidylcholine and phosphatidylethanolamine are the major components (14, 15). A significant proportion of the phospholipid in sperm membranes is in the form of plasmalogen. This is quite a 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 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 (16), i.e. other cells in which membrane transduction signals and cell to cell interaction are fundamental to physiological functions. In this connection, it has recently 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 (17).

2.2. The polyunsaturated fatty acids

The analysis of the fatty acid pattern of membrane phospholipids and plasmalogen has shown the presence of significant amounts of polyunsaturated acids. PUFA are known to contribute to membrane fluidity and flexibility (18-20). The fatty acid composition of cell membranes regulates the activity of different lipid dependent membrane-bound enzymes, again including the second messenger systems and membrane resistance to physical and chemical stress. In ram sperm membrane an asymmetric transversal distribution of phospholipids has been demonstrated with aminophospholipids preferentially located in the inner leaflet and choline-containing phospholipids in the outer. In this connection, the presence of an aminophospholipid translocase in the membrane and fluorescence study results suggest a transbilayer movement of phospholipids and the importance of their transversal segregation in the fusion process during fertilization (1, 21).

The three families of PUFA are classified according to the distance of the first double bond to the methyl terminal, i.e. n-3, n-6 and n-9. Alpha-linolenic acid (C18:3 n-3), linoleic acid (C18:3 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 alpha-linolenic acid (C18:3 n-3). These di-unsaturated fatty acids are normally assumed via the normal diet and are converted into their long chain derivatives by a series of elongation and desaturation. This metabolism takes place mainly in the liver as desaturase activity has been detected almost exclusively in this organ. Delta-6-desaturase is considered a rate-limiting enzyme. The lipid composition of hepatic microsomes and the presence of vitamin E and selenium, which could act as co-factors of this enzyme (22), probably regulate its activity. Furthermore, PUFA are the precursors of prostaglandins and leukotrienes, important factors in both sperm motility and inflammatory processes. In particular, prostaglandin E and 19-OH PGE have been shown to be related to sperm motility. The definitive lipid pattern of ejaculated spermatozoa is reached only after epididymal maturation. This was first demonstrated with ram testicular and ejaculated sperm, which showed different lipid patterns (23, 24). A complete rearrangement of the pre-existing membrane structure during the sperm passage through the epididymis, both for proteins and lipids, has been shown in rams (25), guinea pigs (10, 26) and rats (1, 27, 28). It is conceivable that, in humans too, biomembrane fluidity can increase parallel with the increase of the degree of unsaturation (associated with the fatty acyl component of phospholipids) when sperm pass from the caput to the cauda of the epididymis. This would indicate an active lipid metabolism of the spermatozoa.

2.3. PUFA sperm membrane composition

By means of combined gas chromatography mass spectrometry (29) we studied the PUFA membrane

constitution in human ejaculated spermatozoa. Spermatozoa were examined after washing. High levels of PUFA were detected higher than those detected in blood serum phospholipids. This higher percentage of PUFA has already been noted in humans (30, 31) and also in mammalian spermatozoa by other Authors (see 15), but without a proposed relation with sperm fertilizing ability. However, significant differences in the percentage have been observed depending on the type of the diet and, in animals, on the season. These unusual findings suggest an active fatty acid metabolism and desaturation either during spermatogenesis or during epididymal sperm maturation.

We studied for this purpose, by means of an ad hoc modified Percoll gradient (32), sperm populations and immature germ cells (IGC) present in the semen of the same subjects. In whole spermatozoa we found that the percentage of saturated fatty acids was around 50%, palmitic (C16: 0) and stearic acids (C18: 0) being the most representative. Oleic acid (C18:1 n-9) was the principal monounsaturated fatty acid and both the essential fatty acids linoleic (C18:2 n-6) and linolenic acid (C18:3 n-3) were present. The concentration of the oleic acid resulted significantly higher. Among the metabolites deriving from the desaturase activity, detectable concentrations were observed of C 20:3, C20:4 and C22:4 (belonging from n-6 series) and of C20:5 and C22:6 (from n-3 series). The total percentage of these PUFA was around 30% and among these n-6 PUFA represented 1/3 and n-3 series 2/3. Docohexaenoic acid (DHA, C22:6 n-3) was the most representative among the PUFA. Significant differences were found in the percentage distribution of fatty acids in the sperm populations and IGC separated on Percoll gradient. In the sperm populations, the percentage of saturated fatty acids was not significantly modified, whereas in the unsaturated fatty acids a significant rearrangement was observed. Evaluating linoleic acid (C18:2 n-6) and alpha-linolenic acid (C18:3 n-3), the percentage of these essential fatty acids, decreased in the different Percoll fractions. The percentage of C20:3 n-6, among the desaturase metabolites of the n-6 series, was not significantly modified in the different Percoll fractions. The percentage of C20:4 n-6 progressively decreased and this decrease was associated with an increase of C22:4 n-6. On the contrary, the desaturase metabolites of the n-3 series detected, C20:5 and C22:6 n-3, progressively increased in the higher Percoll-concentrated layers. Evaluating linoleic acid (C18:2 n-6) and alpha-linolenic acid (C18:3 n-3), a significant inverse correlation with the Percoll gradient concentrations was observed. A direct linear correlation was found, on the contrary, between the increase of DHA and those of the Percoll gradient. The high percentage of C22:6 n-3 correlates well with the morphology of the spermatozoa. An inverse relationship was observed in fact between the percentage of atypical forms scored in each layer and the percentages of C22:6 n-3 evaluated in the same layers. The best morphological pattern corresponds to the highest content of this polyunsaturated fatty acid. In comparison with the pattern of mature cells, significant differences were found in the IGC isolated by the second Percoll gradient. As in sperm cells, the saturated fatty acids were mainly represented by C16:0 and C18:0, but the percentage related to the total fatty acids analyzed was significantly higher. Monounsaturated fatty acids were composed of C16:1 n-7 and C18:1 n-9 and the percentage of linoleic acid (C18:2 n-6) and of alpha-linolenic acid (C18:3 n-3) were significantly higher than in mature sperm cells. Among the desaturase metabolites, on the contrary, n-6 PUFA and n-3 PUFA were significantly lower in comparison with those observed in mature sperm cells. Di-homo gamma-linolenic acid (C20:3 n-6), arachidonic acid (C20:4 n-6) and docosahexaenoic acid (C22:6 n-3) were the most represented PUFAs, but they were present at lower percentages if compared to mature sperm cells. These results show that human germ cell line have an active lipid metabolism which produces a rearrangement of the constitution of the fatty acids, causing an elongation and desaturation of the essential fatty acids during the spermatogenesis and possibly also during sperm maturation process (33).

The high percentage of unsaturated PUFA in the membrane of mature and morphologically normal sperm could indicate that spermatozoa are extremely sensitive to external stimuli. The fertilizing function of spermatozoa could explain why this cell is provided with a fragile, but very active membrane that can easily be destabilized and activated. This high unsaturation of PUFA could also provide a biochemical confirmation of the essential role of the sperm plasma membrane in the fertilization process. It is worth stressing that similar percentages of PUFA, and in particular of C22:6 n-3, are detectable in the membranes of nerve cells or cells derived from the neural crest, such as melanocytes (34).

It is interesting to note, in this connection, that rats fed with an essential fatty acid deficient diet can show (together with decreased levels of PUFA in both red blood cells and serum) 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 (35). These alterations and the subsequent infertility are related to the marked reduction in the level of arachidonic acid of the total fatty acid content of the testis.

An alteration in the PUFA constitution of the plasma membrane can be postulated as a common base for sperm pathology in many andrological diseases (varicocele, germ free genital tract inflammation) and can explain the membrane's increased vulnerability after exposure to reproductive toxic compounds. PUFA are in fact one of the main targets of free radical damage and an inverse relationship between lipid peroxides and sperm motility has been clearly demonstrated (36) in vivo and in vitro.

3. ENZYMATIC AND NON-ENZYMATIC SCAVENGER SYSTEMS AND SPERM LIPOPEROXIDATION

3.1. Preliminary remarks

Reactive oxygen species (ROS) are chemical species, endowed with an unpaired electron, which reacts

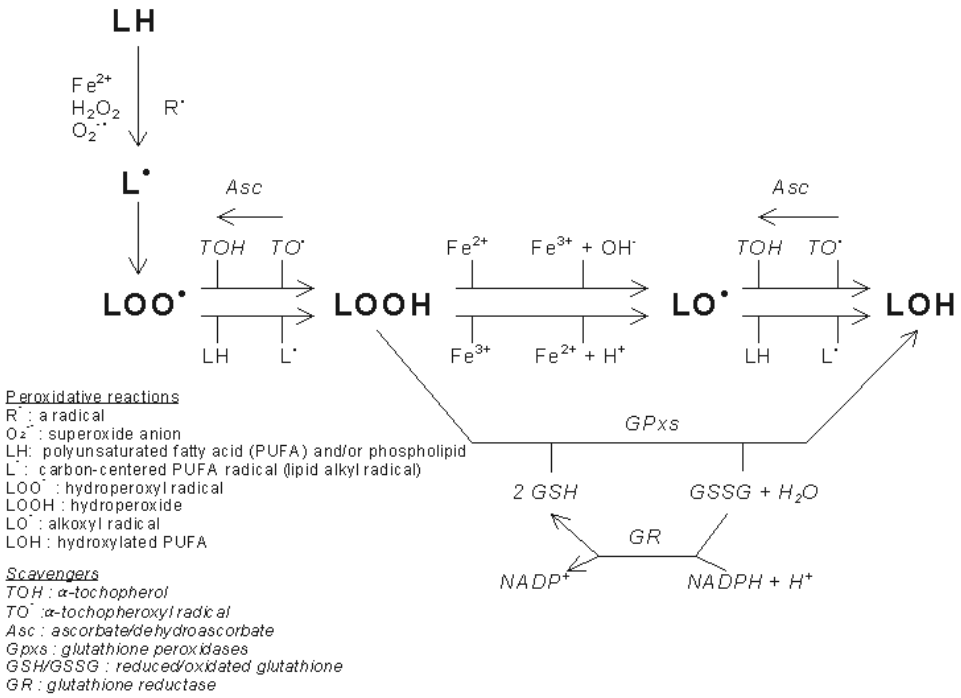

Figure 1. Scheme of lipid peroxidative and scavenging reactions.

promptly with both other free radicals and non-radical molecules, thus triggering a spreading radical reactions sequence (37). The figure 1 summarizes the main peroxidative reactions on PUFA, the glutathione-dependent enzymatic scavenger mechanism and a non-enzymatic one, based on vitamine E and C. A recent review by Ochsendorf (38) illustrates exhaustively the up-to-date state of the art on the ROS and the male genital tract.

The single electron reduction, with the consequent generation of the superoxide anion radical, hydrogen peroxide and the hydroxyl radical, is more frequent than the two-electron reduction, owing to their parallel electron spin arrangements. ROS are very unstable in biological material: they are highly reactive and the hydroxyl radical is considered the most powerful. It can cause effective biological damage only if generated in close proximity to a potential target molecule or directly at the critical cellular target site. Hydroxyl radicals are generated for example during the respiratory burst of neutrophils and macrophages and are the cytotoxic products of the less toxic superoxide anion radicals and hydrogen peroxide. Degradation of lipid hydroperoxides by the reaction with superoxide anion radicals or the reaction of hydrogen peroxides with transition metals leads to the generation of hydroxyl radicals too. Superoxide anion radicals are also generated in mitochondria in normal conditions. Increased production can occur during altered electron transport of mitochondria, microsomes and plasma membranes, as in the case of tissue hypoxia-ischemia. In the micro-environment of cell membranes, the formation of hydroperoxides from superoxide anion radicals is favored.

The hydroperoxyl radical, which is a more powerful oxidant, may be the predominant form of superoxide anion in phospholipid membranes and it is able to peroxide PUFA, thus initiating a chain reaction.

Hydrogen peroxide is the most stable intermediate of oxygen reduction. It is generated during the univalent reduction of oxygen mainly by the xanthine/xanthine oxidase system. For this reason, sperm damage can be induced in vitro by pentoxifilline or other xanthines if these substances are added to the culture media without due attention to sperm characteristics, time of incubation and concentration. In addition, different cells involved in the inflammatory processes can produce hydrogen peroxide and it crosses intra and extra-cellular membranes almost as freely as water. Hydrogen peroxide is cytotoxic and a weak oxidant agent, which in the presence of transitional metals such as iron or copper produces hydroxyl radicals by the Fenton reaction.

It should be pointed out, however, that there is a growing body of evidence indicating that low amounts of ROS are responsible for the physiological control of some sperm functions (39, 40).

3.2. Enzymatic and non-enzymatic scavenger systems in spermatozoa

The levels of peroxidable substances, such as PUFA, and the levels and the activity of the free radical scavenger systems generally regulate cellular homeostasis. Therefore, an oxidative stress can be defined as any disturbance of the balance between pro-oxidants and anti-oxidants, with the former prevailing.

Table 1. Specific activities in rat epididymal spermatozoa

Sperm source	SOD°	CAT+	GR*	PHGPx*	GPx*
Whole epidid	0.59±0.11	7.54±1.18	1.40±0.18	1.58±0.39	4.40±0.88
Caput epidid	0.76±0.26	8.72±1.60	1.49±0.24	3.32±0.53	8.09±1.02
Cauda epidid	0.44±0.09[c]	5.30±2.80[d]	1.33±0.23[d]	0.95±0.27[a]	3.41±0.38[b]

mean ± SD of 5-10 separate measurements, * nmol NADPH/min.10^6 cells, + nmol O_2/min.10^6 cells, °U/10^6 cells, [a]$p<0.0005$, [b]$p<0.005$, [c]$p<0.05$, [d]not significant, in comparison to the caput epididymis spermatozoa corresponding value

Table 2. Solubilization (%)* of PHGPx from rat testis cells and spermatozoa

	KCl	2-ME	KCL + 2-ME	Triton x100
Whole testis nuclei	48[a]	33[a]	74[a]	22[b]
Sperm heads	0	23[b]	27[a]	0
Whole testis mitoch.	5[c]	3[d]	19[b]	22[b]
Sperm mitochondria	0	18[b]	21[b]	3[d]

*ratio (%) of soluble PHGPx activity (mean of 5 experiments) over total (soluble + bound) PHGPx actvity (mean of 5 experiments); controls: untreated samples, [a]$p<0.001$, [b]$p<0.01$, [c]$p<0.05$, [d]not significant, as compared to the untreated samples

The studies of the processes resulting from the action of these two competing agents hold a paramount relevance over the complex sequence of events from the spermatogenesis to the sperm-oocyte fusion. The mammalian testis metabolism aimed to the proper defense mechanism against the (lipo)peroxidative stresses has been considered over many years (41, 42) by examinating both the enzymatic and non-enzymatic scavenging systems capable of assuring a correct spermatogenesis and spermiogenesis. Enzymatic activities have been considered (catalase, superoxide dismutase, peroxidases), as well as the presence of molecules (tochopherol, ascorbate, carotenoids, quinones, GSH) and elements such as selenium. Se deficiency has long been recognized as one of the major impairing factors of physiological male germ cells maturation (41, 43-47). Se-dependent proteins and/or enzymes have therefore been actively considered and characterized in mammalian reproductive tract (48, 49). Details concerning testis oxidative stress mechanisms and defense lie outside the subject discussed here. We will therefore consider some aspects of these phenomena, based on the above mentioned scavenger molecules on the rat spermatozoa, on the basis of the most recent experimental data and knowledge.

3.2.1. Enzymatic scavenger systems

The transit of mammalian spermatozoa from caput to cauda epididymis is a sequence of rather complex maturation processes, mainly mediated by the epididymal environment. The ultimate result is the transformation of a non-motile, non-fertile cell into a potentially fertile one. All these transformations chiefly involve chemical and physical modifications of the lipid assemblage of the plasma and outer acrosomal membrane (1, 50-53). These changes produce a membrane architecture that, as already noted, is potentially able to fuse ("fusogenic"), but is still prevented from doing so. Maturing spermatozoa therefore need specific mechanisms to protect themselves against peroxidative damage of the PUFA-rich membranes and ultimately of the functions regulated by them. Human spermatozoa exhibit both spontaneous lipoperoxidation (54) and a well balanced antiperoxidative capacity just sufficient to accomplish fertilization (55). μM H_2O_2 produced in vitro on human spermatozoa appears to be the most toxic ROS owing to its capacity to cross membranes, to inhibit glucose 6P dehydrogenase, GPx, SOD and to decrease PUFA concentration (56). Data available for epididymal sperm cells indicate that they are equipped with enzymatic activities, which can dispose of H_2O_2 and other potentially harmful ROS. Superoxide dismutase (SOD, E.C. 1.15.1.1), GSH peroxidase (GPx, E.C.1.11.1.9, referred to as GPx1 or the "classical" Se-enzyme GPx, tetrameric) and GSH reductase (GR, E.C. 1.6.4.2) are reported to be certainly present in epididymal spermatozoa (see 57 for a survey). The catalase (CAT, E.C. 1.11.1.6) activity is, on the contrary, still a matter of debate (58-62), presumably owing to the different degree of purity of the sperm cells preparation. Phospholipid hydroperoxide GSH peroxidase (PHGPx, E.C. 1.11.1.19, referred as GPx4, the 20 kDa monomeric form of the Se-enzymes GPxs) (63) was recently monitored for the first time by us in epididymal sperm cells (64). PHGPx specifically reduces in situ the phospholipid hydroperoxides, which can be produced by ROS actions, into the harmless corresponding alcohols, thus interrupting the radical cascade leading to deeper membrane alterations.

Table 1 reports the indicated enzymatic activities in pure isolated epididymal spermatozoa (57). There is a significant statistical difference between caput and cauda epididymal sperm cells for PHGPx, GPx and SOD, which display activities about 2-3 fold higher in the caput sperms.

The binding of PHGPx to the different epididymal sperm cell subfraction was tested by us and compared with similar data collected for whole testis nuclei and mitochondria (64, 65). Table 2 reports the values, expressed as percentages of solubilized PHGPx activity, obtained from whole testis isolated nuclei and mitochondria, and from purified sperm heads and mitochondria. The agents used were ionic strength (1M KCl), a thiol-reducing agent (5mM 2 mercaptoethanol: 2-ME) and a non-ionic detergent (1% Triton X-100).

These data indicate substantial differences in the way the enzyme is bound to the mitochondrial membranes and the chromatin structure of the germ cells or to the maturing spermatozoa. PHGPx is generally more

firmly bound to mitochondria, irrespective of their very different structure and assembly, and better anchored to sperm head chromatin than to testis cells' nuclei. The thiol groups reducing compound 2-ME and, more, its effect combined with the ionic strength elicit the highest detachment in all the subcellular domains. The solubilization produced by 2-ME may be linked to the fact that, while maintaining PHGPx in the reduced form, it probably removes disulfide interaction in the enzyme itself or with other groups anchoring it, e.g., to chromatin structure, where 2-ME is indeed more effective. The non-ionic detergent, whose main target is the membrane structure, is ineffective when the membranes are assembled in more complicated ways (sperm head, sperm mid-piece mitochondria), than in simpler cells (testis nuclei, testis mitochondria).

The presence and distribution in testis cells and spermatozoa of PHGPx, among other enzymes, seems to be quite relevant for a number of different reasons. It is remarkable that the testis exhibits the highest specific activity of PHGPx so far monitored in several tissues: up to two orders of magnitude higher than that of, e.g., brain or liver (66, 67). The enzyme activity in testis is moreover gonadotropin-dependent, and expressed in testis only after puberty (66, 68, 69). Recent results suggest additional roles for PHGPx other than the established antioxidant function. In different somatic cells PHGPx is, in fact, involved in inactivation of 5-lipoxygenase by lowering of the level of the fatty acid hydroperoxides required for the full functioning of the lipoxygenase, thus resulting ultimately in a modulation of leukotrienes (70, 71). A similar phenomenon has been demonstrated for purified 15-lipoxygenase (72). In this new and open context we have recently collected results on the distribution and functions of PHGPx in rat testis cells, spermatozoa and their subfractions, on the basis of both enzymatic and immunochemical approaches. Data obtained clearly indicate that the enzyme presence begins in testis spermatocytes, both in mitochondrial membranes and in the nucleus, where it is for the major part firmly bound (65, 67). The PHGPx activity persists, although at different degrees, during the following phases of the spermiogenesis and of the maturative process of the spermatozoa in the epididymal transit (table 1). The major localizations are still the mitochondria (including those, transformed into the peculiar crescent shape, which surround the sperm cell mid-piece), the high condensed chromatin of the head of the spermatozoa, and the acrosomal membranes since their formation in the round spermatides (64). The intriguing novelty in the functional ambit concerns the ability of PHGPx to oxidize thiol groups other than that of GSH or other small molecules. We demonstrated, in fact, that the enzyme specifically oxidizes the reduced –SH groups of isolated epididymal caput sperm protamine (64). This result is of great interest, if we consider that the hystones are progressively substituted during the spermiogenesis by the unique sperm-proteins protamines. In mammals these proteins are rich in cysteine residues, whose oxidation is crucial for the correct assembly and condensation of the mature sperm cell chromatin (73). The protamine-dependent compactness and resilience of the DNA make it particularly resistant to DNase action, thus protecting it until the proper enzymatic events allow the decondensation and the fusion with the oocyte DNA. From this point of view the selective PHGPx activity towards the caput protamines thiols constitutes an exciting new starting point for an additional regulatory function. If one considers (table 1) the greater amount of the enzyme in epididymal caput spermatozoa, it could be conceivable to envisage a key function of PHGPx in the oxidation of the caput protamines thiol groups. The disulfide bond formation and the chromatin condensation are already concluded in the cauda epididymis, and this could well correlate with the parallel decrease of PHGPx activity there (table 1).

On the whole these new roles can be viewed as a wider capability of the enzyme to modulate a "peroxide tone" via the regulation of the status of endogenous hydroperoxides and endogenous thiol-bearing molecules, as already envisaged by some Authors (71, 74), also for the DNA compaction mechanism (75).

The very recent additional function of PHGPx, in the midpiece mitochondria of the mature rat spermatozoa, seems to be that of a structural molecule, as an enzymatically inactive, oxidatively cross-linked, insoluble protein (76). This new role may explain the Se-deficiency-dependent alterations of spermatozoa.

3.2.2. Non-enzymatic scavenger systems

Close to the enzymatic activities, of which PHGPx is of course not the only one to be considered, non-enzymatic systems are to be examined in strict connection.

The couple GSH/GSSG is, e.g., one of the most important non-enzymatic molecules, at least if one considers the presence of the above mentioned GSH-dependent enzymes. Some Authors indicate that the production of GSH in spermatogenic cells is dependent upon Sertoli cells and is based on the interaction between different cell types (77, 78). The amount of GSH sperm cells is contradictory referred to in literature: the data range from complete absence (79) to highly variable amounts in different specimen (80). In principle, the presence of GSH in sperm cells should be expected, although for PHGPx some other thiol-containing substrates may exist. The limited amount of GSH, the sensitivity and accuracy of the assay and the purity of the sperm cell preparations are once more the main reasons which can explain the discrepancies in the detection. Our data (57) indicate in rat a value of about 35 pmol GSH/10^6 sperm cells, without appreciable differences between caput and cauda epididymis spermatozoa. The value is similar to those reported for goat, rabbit, ram, dog, boar and human specimens (80). The reducing equivalents necessary for regeneration of GSH by GR are provided by NADPH and glucose 6P dehydrogenase activity, which has been demonstrated to be the limiting step in the overall reductive pathway (81).

Alpha-tochopherol and ascorbate are two other fundamental scavenger molecules capable of interrupting the radical propagation. The lipophylic nature of tochopherol and the water solubility of ascorbate therefore

Table 3. alpha-tochopherol content in rat epididymal spermatozoa

	alpha-tochopherol content
Whole epididymis spermatozoa	3.418±1.220[a]
❑ Heads	0.924±0.007[a]
❑ Tails	1.319±0.035[a]
❑ Plasma and acrosomal membranes	351.115±8.355[b]
Caput epididymis spermatozoa	8.261±1.786[a]
Cauda epididymis spermatozoa	2.175±0.375[a,c]

mean ± SD of 3-12 separate measurements (HPLC and fluorometric detection), [a]pmoles/10^6 cells (or 10^6 heads, or 10^6 tails), [b]pmoles/mg protein, [c]$p<0.0005$, in comparison to caput epididymal spermatozoa

provide protection in all the cellular domains. The two vitamins can, moreover, mutually interact, the being capable to regenerating the ROS-oxidated tochopheryl radical.

If one skims through the literature, it seems, however, that for vitamin E there have been privileged studies on its possible therapeutic use in mammalian infertility (82, 83), rather than studies concerning its physiological presence, distribution and role in germ cells and spermatozoa. We collected data concerning the presence of vitamin E in rat testis mitochondria and rat epididymal spermatozoa. The aim of these studies was to fill a gap, to examine vitamin consumption by peroxidation of membranes, and to establish whether alpha-tochopherol co-operates with the enzyme PHGPx. This possibility is based on the fact that the membrane hydroperoxides can be reduced by PHGPx: this interrupts a radical reaction cascade with the result of possibly sparing the vitamin E (for details see 84, 85).

Mitochondrial membranes of testis cells are richly endowed with alpha-tochopherol. The amount of vitamin in isolated, intact mitochondria of 12-14 weeks old rat testes is 0.603±0.04 nmol/mg proteins (85), a value which is three times higher than that, e.g., in liver mitochondria. In immature rat testes (4 weeks old) there are 0.694±0.07 nmol/mg proteins (85). This is in some way interesting, if one considers that PHGPx is absent in immature rat testes (66). This suggests that the testis antiperoxidative mechanism based on alpha-tochopherol has to be considered comparable before and after puberty, contrary to the mechanism based on phosholipid hydroperoxides elimination catalyzed by PHGPx.

Table 3 reports the content and distribution of alpha-tochopherol in isolated rat epididymal spermatozoa. The vitamin is present, at the head level, in the plasma and acrosomal membrane, and its localization in the tails can be attributed to the mid-piece mitochondria. This indicates continuity in the existence of this scavenger from the germ cells to the maturing spermatozoa. More interesting from the point of view of epididymal maturative processes is the finding that cauda spermatozoa contain only about 25% of vitamin E in comparison to the caput sperm cells.

3.2.3. Perspective

The alpha-tochopherol distribution has to be compared with the similar situation of the enzymes PHGPx, GPx and SOD. This does not appear to be a coincidence, and deserves further consideration. It is clear that our results indicate that stronger endogenous protective equipment (enzymes and tochopherol) is built up in epididymal caput spermatozoa, which are still immature, and that this equipment weakens at the cauda level, where the sperm cells are mature. This could appear strange at first glance: but, it suggests a functional difference in the protective processes in the two spermatozoa populations. In this framework other evidence indicates, moreover, that caput and cauda sperm cells react differently to peroxidative damage (57): the resulting decrease in vitamin E is, in fact, always more marked in cauda epididymal spermatozoa, where both PHGPx, GPx and SOD activities and alpha-tochopherol content are lower than in caput.

As far as the putative synergism between PHGPx and alpha-tochopherol is concerned, our data (57, 85) indicate that neither in testis mitochondria nor in the epididymal spermatozoa is the enzymatic activity able to spare the vitamin. This suggests, therefore, that the two systems are metabolically and probably spatially independent.

All these results can be correlated with the already mentioned (1) changes, which occur in the sperm cells plasma and acrosomal membranes during the passage from caput to cauda epididymis (higher PUFA and cholesterol content, higher asymmetry). The sum total of these conditions renders the membranes more susceptible to alterations, provided that the so-called “safety device” (1), represented by cholesterol and membrane proteins, is removed. But this event is far from physiologically probable inside the epididymal lumen. The situation of the cauda epididymal spermatozoa can thus be defined as “potentially fusogenic” or potentially capable of undergoing acrosomal reaction (1, 86). The lower protective equipment inherent in cauda epididymal sperm cells can to be looked at in line with this picture. In the cauda domain the main endogenous scavenger systems are such as to be considered less compulsory that in the caput. Moreover, as spermatozoa enter into the seminal plasma, other scavenger molecules, present there, can assume the protective role (87-89) up until the final event of the fusion with the oocyte.

In conclusion, on the basis of these findings, it is clear that the key domains of maturing epididymal spermatozoa (acrosome, chromatin, mitochondria) are endowed with effective scavenger systems, whose concentration, distribution and binding are in some way finely tuned by the sperm cell itself and/or by the epididymal fluid (90, 91).

3.3. Seminal fluid and ROS damage

In comparison with other cells, it appears that in mature spermatozoa the high concentration of unsaturated lipids is associated with the relative paucity of some oxyradical scavenger mechanisms, owing also to the

virtual absence of cytoplasm in mature sperm cells. This deficiency, 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 SOD, catalase and GSH peroxidases activities together with significant concentrations of antioxidants such as ascorbic acid, tocopherol and GSH (87). The antioxidant system works in an integrated fashion. SOD, which is a metallo-enzyme, dismutates the superoxide anion radical into hydrogen peroxide. Ceruloplasmin can have a similar scavenger effect. The hydrogen peroxide produced during the reactions has to be removed by the action of both catalase or GSH peroxidase (GPx). If the function of these enzymes is insufficient for complete elimination of hydrogen peroxide, the Fenton reaction takes place in the presence of transitional metals with the subsequent production of toxic hydroxyl radicals. Vitamin E is the chain breaking antioxidant in membranes, and its oxidation produces the tochopheryl radical, which can then be reduced by ubiquinone or ascorbic acid. The oxidation of Vitamin C gives rise to ascorbyl radicals which in turn can be reduced by GSH, producing thiyl radicals and oxidized glutathione, which can be regenerated by GSH reductase. As a consequence, the whole system has to work simultaneously and an alteration of one of the components can lead to a potentially damaging accumulation of ROS (92).

One special attribute of the antioxidant systems in seminal plasma is the relatively high concentration of GSH. Its thiolic group can react directly with hydrogen peroxide, superoxide anion, hydroxyl radicals and can react with alkoxyl radicals and hydroperoxides, producing alcohols (14).

In different biological systems, the GSH redox cycle plays an important role in protecting cells against oxidative damage (93-95). Generally, GSH is present in millimolar concentrations in cytosol and the nucleus, whereas its concentration is low in blood serum and other biological fluids (14, 87). The presence of significant concentrations of this reducing agent has been correlated to the liquefaction process of seminal plasma and to 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 level of catalase in spermatozoa and seminal plasma is low, GSH and GSH peroxidase are the main factors that can remove the hydrogen peroxide generated (93).

The harmful impact of radicals and toxic compounds on sperm function has been well studied with particular emphasis on the negative effects of ROS on sperm function (96-99). In mammalian cells the alteration of the fatty acid pattern of the membrane produces significant modifications including changes in the activity of different lipid-dependent enzymes and in the resistance to physical or chemical stress (100).

The risk for spermatozoa to undergo a lipid peroxidation can be assessed using a spectrophotometric assay to test the generation of malonildialdeyde promoted with ferrous and ascorbate irons in the presence of thiobarbituric acid (TBA) (97). TBA evaluates the ferrous iron-catalyzed 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 (101). Many andrological pathologies have been associated with an increase of the lipid peroxidation risk such as varicocele, infections and germ free genital tract inflammation (29). The same pathologies induce many biological and clinical effects, such as modifications in microcirculation, venous stasis and subsequent hypoxia, leucocyte activation and cell necrosis, all of which increase ROS in the semen. In fact, studies have shown radical oxygen species to be higher in the male partner of infertile couples suffering from selected andrological conditions (97, 99, 102).

Particular attention has been given to the harmful effects of ROS on in vitro spermatozoa and the implications of this in semen preparation in In Vitro Fertilization (IVF) programs. However, some reports have shown that ROS can trigger in vitro physiological sperm functions, such as capacitation and hyperactivated motility (103-105). These positive effects are strictly related to the equilibrium between ROS and the scavenger systems. Actually, great differences are observed in the results of sperm selection techniques for assisted reproduction, if the semen characteristics are not taken into account and the sperm preparation methods are not chosen case by case (106). The culture media of preparation and the substances added to it must also be selected bearing the above in mind. For example, the very well studied pentoxifylline has in vitro a stimulating effect 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 (107). Recently, it has beem reported that in oligozoospermic patients the high ROS concentration may also be of intracellular origin (108). This could explain both the fragility of sperm cells of these patients during the in vitro treatment and the inefficiency of antioxidants added to the in vitro colture media in antagonizing the ROS indiced damages (109).

4. SCAVENGER THERAPY: THE ROLE OF GSH

4.1. Preliminary remarks

Given that it has usually proved impossible to identify the true etiology 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, phosphatidilcholine, callicreine, penthossiphylline and vitamins A, E and C (15, 82, 83, 110). Unfortunately, the real difficulty may be that andrologists only get to 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 prescribe drugs to try to improve seminal parameters, theoretically acting directly on gamete production or epididymal maturation. Controversial data have often resulted from the frequently uncontrolled studies carried out to support such therapies.

As already discussed, spermatozoa and seminal plasma have many scavenging systems to counteract the harmful effects of ROS and other toxic compounds. These known systems are, as a whole, enzymes, proteins (albumin), GSH, vitamin E and C, taurine and hypotaurine, and mercapturic acids (111). GSH seems, among the latter, to be one of the most indicated drugs owing to its demonstrated antitoxic and antioxidant action in other degenerative pathologies (cirrhosis, neoplasia, and consequences of antiblastic therapies). In the past, sulfydryl constituents of semen (cystein, GSH and ergothioneine) have been shown to play an important role in maintaining sperm motility and metabolism in experimental conditions (15) and, in isolated rat spermatides, GSH can prevent the damage due to exposure to peroxidizing agents (112). The pharmacology of exogenous administration of GSH has yet to be completely studied and it is unlikely that the polar molecule can cross the cell membranes. However, it has been shown in experimental animals that GSH administration reduces stress induced gastric injury without any modification of the tissue concentration of antioxidant (93).

4.2. GSH therapy

One of the most important points in selecting GSH as a therapeutically agent relies on its physiologically significant presence in seminal plasma. Even though it cannot cross cell membranes, after systemic administration the antioxidant can increase its concentration in biological fluids; it can reach the seminal plasma and can concentrate there, thus exerting its physiological and therapeutically role (113).

For all these reasons we have developed a research line dedicated to the use of GSH in sperm pathology and to its mechanism of action.

We started (114) a two-month pilot study on GSH (600 mg/day i. m.) in a small group of eleven patients with dyspermia associated with chronic epididymitis (2 cases), prostate-bladder germ-free chronic inflammation (6 cases), varicocele (2 cases), and antisperm antibodies (1 case). We carried out standard semen analysis (115) and a computer analysis of sperm motility before treatment and also after 30 and 60 days of therapy. GSH therapy had a statistically significant effect on sperm motility patterns and sperm morphology. In particular, a significant difference between baseline and 60-day checks was observed in the percentage of forward motility and in the parameters of the sperm motility computer analysis (velocity, linearity, ALH, and BCF). A significant reduction in the percentage of atypical forms was also seen. Sperm motility improved, especially in patients with chronic epididymitis and patients with varicocele. Good results were also observed in four of six patients with prostate-vesicular chronic inflammation. No modifications were noted in semen parameters or in antibody titer in the case with antisperm immune pathology (114). The results encouraged us to extend the therapy to a wider group of patients, selecting the andrological pathologies and using a controlled trial.

Then we (113) carried out a placebo controlled double blind cross-over trial on a selected group of twenty infertile patients, ten with unilateral varicocele and ten with germ-free genital tract inflammation. The seminological studies we carried out monthly were the same as the pilot study i.e. microscopic semen analysis and computer sperm motion analysis. All the patients were followed up for a wash out period of two months. After this period, the patients received either GSH or placebo for two months, and then they crossed over to the alternative treatment for a further two months. The patients were randomly and blindly assigned to treatment with one i.m. injection every other day of either GSH 600 mg or an equal volume of a placebo preparation. The clinical selection criteria were very strict; i.e. two years of infertility (with a gynecological normal partner); age range 20 - 40 years; no systemic or hormonal pathologies; no history of cryptorchidism; no testicular hypotrophy; no detectable genital infections; no antisperm antibodies and, above all, strict seminological inclusion criteria. The group was homogeneous for sperm concentration, motility and morphology at the start of the trial. No variations were seen in semen volume and leukocyte concentration. The effect of GSH (table 4) was already significant after one month of treatment on the sperm variables. It continued (even though gradually decreasing) one and two months after the end of therapy during the placebo period. All twenty selected patients showed a significant increase in sperm concentration and a highly significant improvement in sperm motility, sperm kinetic parameters and sperm morphology. The improvement was significant not only from a strict statistical standpoint, but also from a biological and clinical point of view. There were no significant differences between patients suffering from varicocele or germ free genital tract inflammation. In these pathologies, the production of ROS can play a pathogenetical role both in the sperm cell membrane and the metabolic alterations of spermatozoa leading to hypomotility and teratozoospermia. This could explain the pharmacological effect on sperm motility and kinetic patterns. Moreover, the significant reduction in percent of atypical forms seems to indicate a more complex action than a single metabolic effect. One possible explanation could be that GSH affects sperm membrane maturation and the biochemical constitution of the cell. This effect has to be a post-spermatocyte action as the period of therapy was specifically chosen to be shorter than a complete spermatogenetic cycle and because earlier positive results were seen after the first month of treatment. These effects on sperm motility and morphology lasted beyond the therapy period and this suggests that GSH also acts indirectly by the improvement of GSH peroxidases metabolic condition of the testicular-epididymal environment. Finally, the slight improvement observed in sperm concentration could be explained by a reduced sperm phagocytosis at a post-testicular level due to better sperm

Table 4. Sperm parameter variations during the placebo cross-over trial of glutathione therapy in male infertility

	Sperm Concentration	Total motility	Forward motility	Morphology	Velocity	Linearity	ALH	BCF
	10^6/ml	%	%	% atypical	μ/sec	Index 1-10	μ	Hz
Base	26.35 ± 12.64	28.00 ± 10.37	12.00 ± 5.20	57.20 ± 8.72	28.03 ± 3.55	2.70 ± 0.35	2.48 ± 0.20	11.09 ± 0.47
Glu I	30.20 ± 10.73	38.50 ± 10.55	26.50 ± 8.51	48.10 ± 7.52	42.80 ± 5.31	4.30 ± 0.69	3.55 ± 0.25	12.93 ± 0.40
Group 1								
Glu II	29.80 ± 10.64	38.00 ± 11.60	27.50 ± 8.58	48.20 ± 7.84	43.70 ± 5.40	4.35 ± 0.67	3.52 ± 0.30	12.84 ± 0.44
Pla I	30.00 ± 19.71	32.50 ± 12.96	20.00 ± 8.82	50.8 0 ± 5.22	38.80 ± 4.85	3.79 ± 0.85	3.35 ± 0.30	12.64 ± 0.47
Pla II	28.20 ± 11.63	28.50 ± 12.70	18.50 ± 8.83	53.40 ± 7.57	36.10 ± 5.69	3,50 ± 0.89	2.98 ± 0.23	12.25 ± 0.48
Base	27.56 ± 15.69	24.83 ± 9.70	10.50 ± 4.30	63.20 ± 6.62	28.80 ± 1.91	2.69 ± 0.44	2.55 ± 0.17	11.12 ± 0.59
Pla I	27.40 ± 14.10	26.00 ± 9.07	10.50 ± 2.84	62.50 ± 6.19	28.60 ± 3.90	2.61 ± 0.32	2.44 ± 0.22	11.05 ± 0.58
Group 2								
Pla II	28.10 ± 13.10	26.50 ± 10.29	10.00 ± 4.71	62.50 ± 6.38	28.70 ± 2.63	2.64 ± 0.47	2.53 ± 0.17	11.10 ± 0.56
Glu I	31.40 ± 13.64	35.50 ± 4.38	28.50 ± 4.12	52.10 ± 5.61	47.20 ± 4.94	4.78 ± 0.59	3.67 ± 0.31	12.98 ± 0.45
Glu II	31.10 ± 13.40	37.00 ± 4.83	29.50 ± 4.97	52.40 ± 6.47	47.10 ± 5.74	4.78 ± 0.60	3.67 ± 0.35	12.96 ± 0.47

Base : Mean and SD of the three seminal analyses of the wash out period , Glu I : Mean and SD after 1 month of glutathione therapy , Glu II : Mean and SD after 2 months of glutathione therapy , Pla I : Mean and SD after 1 month of placebo. , Pla II : Mean and SD after 2 months of placebo

structure. No side effects or significant modifications in hormonal patterns were noted in the patients.

The therapeutic use of GSH (and SOD) has also been proposed for cases with oligozoospermia, to avoid possible sperm damage induced by the contact of healthy sperm with pathological components of the semen during in vitro manipulation for assisted reproduction (104).

To study the possible in vitro action, we tested GSH directly 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 leucocyte-produced ROS (116, 106). We used microscopic semen analysis and computerized sperm motion analysis and tested the sperm parameters on basal semen and on post-rise spermatozoa obtained by layer and pellet-swim up in Tyrode's solution (TIR) alone or containing 1mg/ml of GSH. In cases of leucospermia, we studied in vitro GSH on basal semen, diluted semen (1:1 semen and TIR + GSH) and post rise spermatozoa after layer and pellet-swim up. The results showed no significant differences between sperm parameters of basal semen and semen diluted with TIR+GSH media; no significant differences between layer swim up with and without GSH in the media were detected. The only significant difference was found in sperm forward motility in leukospermic samples treated with pellet swim up. In these samples an increase was found when there was GSH in the migration media (117). These results indicate that GSH also protects sperm motility during pelletting, where there can be contact between seminal ROS produced by leukocytes or damaged spermatozoa.

4.3. PUFA as markers of sperm pathology: a rationale for GHS therapy success

In order to explain the above results we started to explore the possible mechanisms through which GSH acts on the sperm function. As previously stated, oxidative stress may be defined as any imbalance between pro-oxidants and anti-oxidants in which the former prevail and produce a free radical cascade leading to a lipoperoxidative process. So PUFA (PUFA) of phospholipids play a major role in membrane constitution and function and are one of the main targets of the lipoperoxidative process. Therefore, to understand the therapeutic action of GSH, we strictly selected infertile patients with unilateral varicocele and germ free genital tract inflammation and studied the modifications produced by the therapy in a) semen variables and lipoperoxidation sperm membrane risk (evaluated by TBA assay) and b) on the pattern of fatty acids of phospholipids from blood serum and red blood cell membranes. Red blood cells, in fact, are considered to be a representative model of the constitution of general cell membranes and we used these, as it was practically impossible to evaluate the fatty acid pattern of sperm membrane phospholipids in patients with severe oligozoospermia.

Our study showed that sperm concentration, motility, morphology and kinetic variables improved in selected infertile patients. This increase was already significant after 30 days, indicating that GSH therapy acts on the epididymis, favoring sperm maturation and hampering sperm reabsorption. These improvements were associated with an increase of the reed blood cell levels of PUFA of phospholipids and with a decrease in the levels of lipoperoxides in the sperm (table 5).

This suggested that, at least in part, the therapeutic action of GSH is due to its protective effect on the lipid components of the cell membrane. It is likely that GSH acted as a free radical scavenger (both enzymatically and directly) in the epididymis, thus reducing the lipoperoxidative process generated by vascular or inflammatory pathologies. The significant reduction in the lipoperoxide level in seminal plasma as detected by the TBA test supports this hypothesis (table 5). Moreover, the increase in the sperm cell concentration after one month of therapy (without variation in semen volume) indicates a reduction in the sperm damage and subsequent epididymal reabsorption rather than a variation in spermatogenesis (29). This appears to confirm experimental data that show

Table 5. Lipoperoxidative potential (TBA assay) in spermatozoa of patients studied before (T0) and at the end (T60) of glutathione therapy

	Mean	SD	SE
T 0	14.98	1.07	0.48
T 60	11.84	0.71	0.32
	t = 9.358		
	sig ≤ 0.001		

Mean, standard deviation (SD), standard error (SE), Students t test (t) and significance probability (sig), Values are expressed in nmoles MDA/10^8 spermatozoa.

that rat spermatids utilize GSH-dependent mechanisms against oxidative stress (112, 65). The above results suggest 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 ROS generated in the epididymis following vascular or inflammatory processes, facilitating dyspermia. These results suggest that biochemical modifications in lipid membrane constitution could explain the seminal results of GSH therapy. On the other hand, it seems likely that subjects 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. This could confirm experimental data on rats fed with poor dietary regimen that exploit PUFA deficiency in serum and semen (35, 118).

To confirm this last hypothesis, we selected two small groups of patients suffering from unilateral varicocele, associated or not with dyspermia (named Var-inf and Var-norm), and a control group (named Control) of healthy fertile subjects (table 6). Results show a significant difference between the PUFA of red blood cell membranes of the Control group and the Var-norm patients. On the contrary, there is a significant decrease of PUFA unsaturation in the reed blood cells membranes of the majority of Var-inf patients (119). It is striking that varicocele patients with normozoospermia have PUFA in all the cell membranes similar to those of control subjects and, on the contrary, dyspermic varicocele patients have a significant deterioration in this parameter. It is possible to hypothesize a pathogenetical relationship between the male gamete damage and a lower resistance to ROS aggression against the sperm plasma membrane and the vascular pathology of the testicular-epididymal region. If these data are confirmed with a long-term follow up study, it will become possible to establish a predictive index on the risk of varicocele induced dyspermia; on the basis of this predisposition surgery could then be recommended only to young patients with PUFA alterations in their red blood cell or other cells representative of the general status.

5. CONCLUDING REMARKS

Sperm cells possess an active lipid metabolism and the high levels of membrane PUFA, the significant concentration of free fatty acids in seminal plasma together with the concentration of carnitine are clear evidence that lipids are important sources of energy and are a structural requirement of sperm cells.

The high content of PUFA on the one hand and the need for protection of the highly specialized membrane domains on the other, demand adequate scavenger systems, e.g., during the long migration and maturation of sperm cells in the epididymal tract. These antioxidant enzymatic and non-enzymatic systems are highly diversified and mostly based on the GSH/GSSG couple. Emerging evidence suggests intriguing new roles for some enzymes (such as PHGPx), which can go beyond the merely protective role. The phenomenon of peroxidation in maturing sperm cells is now considered from two point of view, depending on the amount of ROS present at a given time. It is well recognized that ROS are responsible, at least at low concentration, for the physiological control of some sperm functions (39). The studies concerning enzymatic systems involved in ROS production/elimination should be viewed also in terms of regulatory activity, in addition to the simple protective function. The role of these systems in the maturation of sperm cells is to be examined in a new light as the possible instrument to fine tune the “best fit” conditions. As an example, the specific oxidation of epididymal sperm protamines, an additional assignment for common scavenger activity of PHGPx, can therefore be the first sign of this new metabolic role.

In this sense seminal plasma must be regarded as an excellent nutritive and protective medium for sperm cells, on the basis of the fact that it possesses higher concentrations of antioxidants than other biological fluids or blood serum. It becomes aggressive only when pathologies alter its anti- / pro-oxidant equilibrium or when the sperm membrane is more fragile or weaker after in vitro manipulation. Scavengers such as GSH can therefore be used to treat these cases, as they can restore the physiological constitution of PUFA in the cell membrane.

Sperm membrane PUFA composition and its disequilibrium seem to be a sensitive marker of both sperm membrane function and increase in fragility. In particular it would be extremely useful to demonstrate whether the high percentage of n-3 PUFA (namely C22:6 n3) in the sperm membrane is necessary for fertilizing capacity and if a correlation exists between the systemic lipid metabolism and sperm cell functions.

Considering the metabolic pathway of essential fatty acids, our results on the differences between immature germ cells and various sperm populations could mean that long chain PUFA are actively produced also during the maturation of sperm cells after testicular release. The high concentration of carnitine in the human epididymis could support the hypothesis of a post-testicular metabolism of fatty acids as shown in other mammals (120). However, it is also possible to postulate an intra-testicular production of spermatozoa with different degrees of maturation, deriving from a spermatogenesis producing sperm with different PUFA insaturation. Finally, post-testicular peroxidation of

Table 6. Percentage of fatty acids in red blood cells of controls subjects (Contr) and varicocele (Var) patients splitted in Norm and Inf

	Contr	**Var - norm**	**Var - inf**	**Control vs Var - norm**		**Var – norm vs Var - inf**	
	m ± sd	m ± sd	m ± sd	t	sig	t	sig
C16:0 Palmitic	23.60 ± 0.26	25.42 ± 2.41	28.45 ± 4.80	-5.367	0.001	-1.715	0.108
C18:0 Stearic	14.71 ± 0.36	20.7 ± 2.05	22.9 ± 3.00	-19.864	0.001	-1.728	0.106
C18:1 Oleic	25.54 ± 0.95	22.71 ± 2.51	27.4 ± 3.80	6.304	0.001	-2.961	0.010
C18:2 n6 linoleic	9.78 ± 0.94	12.95 ± 2.20	13.04 ± 4.50	-5.737	0.001	-0.055	0.957
C20:3 n6 di-homo-γ-linolenic	1.37 ± 0.09	1.99 ± 0.50	0.86 ± 0.40	-8.402	0.001	4.424	0.001
C20:4 n6 Arachidonic	15.13 ± 0.95	15.49 ± 2.92	6.70 ± 2.50	-0.730	0.468	5.807	0.001
C22:6 n3 Docosahexaenoic	4.10 ± 0.16	3.10 ± 0.70	1.20 ± 0.40	9.297	0.001	5.600	0.001

m ± sd = mean ± standard deviation, t = student t test, sig = probability index

long chain PUFA caused by epididymal micro-environments, which are capable of modifying the relative percentages of PUFA in the different sperm populations, can also be hypothesized.

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7. REFERENCES

1. Nolan JP & R.H. Hammerstedt: Regulation of membrane stability and the acrosome reaction in mammalian sperm. *FASEB J* 11, 670-82 (1997)
2. Miescher F.: Die Spermatozoen einiger Wirbeltiere; ein Beitrag zur Histochemie. *Verh Naturforsch Ges Basel* 6, 138-43 (1878)
3. Miescher F. In: Die histochemischen und physiologischen Arbeiten von Friedrich Miescher. Vogel, Leipzig (1897)
4. Koelliker A: Physiologische studien uber die samenflussigkeit .Z. *Wiss. Zool*. 7, 201-7 (1856)
5. Matthews A: Zur chemie der spermatozoen. *Hoppe Seylers Z. Physiol Chem* 23, 399-406 (1897)
6. Sano M.: Phosphatides in the fish sperm. *J Biochem* 1, 1-25 (1922)
7. Muller CP & G.R.F. Krueger: Modulation of membrane proteins by vertical phase separation and membrane lipid fluidity. Basis for a new approach to tumor immunotherapy. *Anticancer Res* 6, 1181-94 (1986)
8. Yeagle PL: Lipid regulation of cell membrane structure and function. *FASEB J* 3, 1833-42 (1989)
9. Langlais J, F.W.K. Kan, L. Granger, L. Raymond, G. Bleau & K.D. Roberts: Identification of sterol acceptors that stimulate cholesterol efflux from human spermatozoa during in vitro capacitation. *Gamete Res* 12, 183-224 (1988)
10. Myles DG & P. Primakoff: Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *J Cell Biol* 99, 1634-41 (1984)
11. Primakoff P, H. Hyatt & D.G. Myles: A role for the migrating sperm surface antigen PH20 in guinea pig sperm binding to the egg zona pellucida. *J Cell Biol* 101, 2239-44 (1985)
12. Lenzi A, L. Gandini, F. Lombardo, G. Micara, F. Culasso & F. Dondero: In vitro sperm capacitation to treat antisperm antibodies bound to the sperm surface. *Am J Reprod Immunol* 28, 51-5 (1992)
13. Dondero F, A. Lenzi, L. Gandini & F. Lombardo: Study of ASA-reactive-acrosome-antigens from epididymal sperm maturation to in vitro capacitation. *Regional Immunol* 6, 290-97 (1995)
14. T Mann. In: The biochemistry of semen and of the male reproductive tract. Ed: Mann T, Methuen Pub, London (1964)
15. T Mann & C Lutwak-Mann. In: Male reproductive function and semen. Ed: Mann T, Springer-Verlag Berlin (1981)
16. CH Mattews & K.E. Van Holde: Lipid metabolism 2°. In: Biochemistry. The Benjamins-Cummins Pub (1990)
17. DG Cornwell & N Moriski: Lipid peroxides and cell proliferation. In: Free Radicals in Biology, Ed: Pryor W, (1984)
18. Israelachvili JN, S. Marcelja & R.G. Horn: Physical principles of membrane organization. *Quart Rev Biophys* 13, 121-200 (1980)
19. Fleming AD & R. Yanagimachi: Effects of various lipids on the acrosome reaction and fertilizing capacity of guinea pig spermatozoa with special reference to the possible involvement of lysophospholipid in the acrosome reaction. *Gamete Res* 4, 253-73 (1981)
20. Meizel S & K.O. Turner: Stimulation of an exocytotic event, the hamster sperm acrosome reaction by cis-unsaturated fatty acids. *FEBS Lett* 161, 315-8 (1983)
21. Muller K, T. Pomarski, P. Muller, A. Zachowski & A. Hermann: Protein-dependent translocation of aminophospholipids and asymmetric transbilayer distribution

of phospholipids in the plasma membrane of ram sperm cells. *Biochemistry* 33, 9968-74 (1994)
22. Infante JP: Vitamin E and selenium participation in fatty acid desaturation. A proposal for an enzymatic function of these nutrients. *Mol Cell Biochem* 69, 93-108 (1986)
23. Scott TW, J.K. Voglmayr, B.P. Setchell: Lipid composition and metabolism in testicular and ejaculated ram spermatozoa. *Biochem J* 102, 456-62 (1967)
24. Poulos A, P.D.C. Brown-Woodman, I.G.White & R.I. Cox: Changes in phospholipids of ram spermatozoa during migration through the epididymis and possible origin of prostaglandin F2 in testicular and epididymal fluid. *Biochim Biophys Acta* 12, 388-403 (1975)
25. Wolf D.E., S.S. Hagopian, R.G. Lewis, J.K. Voglmayr & G. Fairbanks: Lateral regionalization and diffusion of a maturation-dependent antigen in the ram sperm plasma membrane. *J Cell Biol* 102, 1826-31 (1986)
26. Cowan AE, P. Primakoff & D.G. Myles: Sperm exocytosis increases the amount of Ph-20 antigen on the surface of guinea-pig sperm. *J Cell Biol* 103, 1289-97 (1986)
27. Gaunt SJ, C.R. Brown & R. Jones: Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies. *Exp Cell Res* 144, 275-84 (1983)
28. Hall JC, J. Hadley & T. Doman: Correlation between changes in rat sperm membrane lipids, protein, and the membrane physical state during epididymal maturation. *J. Androl*. 12, 76-87 (1991)
29. Lenzi A, M. Picardo, L. Gandini, F. Lombardo, O. Terminali, S. Passi & F. Dondero: Glutathione treatment of dyspermia: effect on the lipoperoxidation process. *Human Reprod* 9, 2044-50 (1994)
30. Jones R, T. Mann & R. Sherins: Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* 31, 531-7 (1979)
31. Poulos A & I.G. White: The phospholipid composition of human spermatozoa and seminal plasma. *J Reprod Fertil* 35, 265-77 (1973)
32. Gandini L, A. Lenzi, F. Lombardo, R. Pacifici & F. Dondero: Immature germ cell separation using a modified discontinuous Percoll gradient technique in human semen. *Human Reprod* 14, 1022-7 (1999)
33. Lenzi A, L. Gandini, V. Maresca, F. Dondero & M. Picardo: Fatty Acids composition of spermatozoa and immature germ cells. *Human Reprod* (1999) in press
34. Picardo M, P. Grammatico, C. De Luca, S. Passi, M. Nazzaro-Porro: Differential effect of azelaic acid in melanoma cell coltures. *Pigment Cell Res* 20, 142-49 (1990)
35. Leath WMF, C.A. Northop, F.A. Harrison & R.W. Cox: Effect of linoleic acid and linolenic acid on testicular developments in the rat. *Q J Exp Physiol* 68, 221-31 (1983)
36. RJ Aitken: Reactive oxygen species and human sperm function. In: Comparative spermatology 20 years after. Ed: Bacetti B, Raven Press, NY (1991)
37.W Pryor: The role of free radical reactions in biological systems. In: Free radicals in biology. Ed: Pryor W, (1984)
38. Ochsendorf FR: Infections in the male genital tract and reactive oxygen species. *Hum Reprod Update* 5, 399-420 (1999)
39. Griveau JF & D. Le Lannou: Reactive oxygen species and human spermatozoa: physiology and pathology. *Int J Androl* 20, 61-9 (1997)
40. Bize I, G. Santander, P. Cabello, D. Driscoll & C. Sharpe: Hydrogen peroxide is involved in hamster sperm capacitation in vitro. *Biol Reprod* 44, 398-403 (1991)
41. Wu SH, J.E. Oldfield, P.D. Whanger & P.H. Weswig: Effect of Se, vitamin E and antioxidants on testicular function in rats. *Biol Reprod* 8, 625-9 (1973)
42. Robinson BS, D.W. Johnson & A. Poulos: Novel molecular species of sphingomyelin containing 2-hydroxylated polyenoic very-long-chain fatty acids in mammalian testes and spermatozoa. *J Biol Chem* 267, 1746-51 (1992)
43. Behne D, T. Hofer, R. von Berswordt Wallrabe & W. Egler: Se in the testis of the rat: studies on its regulation and its importance for the organism. *J Nutr* 112, 1682-7 (1982)
44. E Wallace, HI Calvin, K Ploetz & GW Cooper: Functional and developmental studies on the role of Se in spermatogenesis. In: Selenium in Biology and Medicine, part A. Eds: Combs GS, Levander OA, Spallholz JE, Oldfield JE, AVI Publ Co., Westport, Conn. (1987)
45. Behne D, H. Weiler & A. Kyriakopoulos: Effects of Se in deficiency on testicular morphology and function in rats. *J Reprod Fertil* 106, 291-7 (1996)
46. Brown DG & R.F. Burk: Se retention in tissues and sperm of rats fed a torula yeast diet. *J Nutr* 103, 102-8 (1973)
47. Wu ASH, J.E. Oldfield, L.R. Shull & P.R. Cheeke: Specific effect of Se deficiency on rat sperm. *Biol Reprod* 20, 793-8 (1979)
48. Mc Connell KP, R.M. Burton, T. Kute & P.J. Higgins: Selenoproteins in rat testis cytosol. *Biochim Biophys Acta* 588, 113-9 (1979)
49. Smith DG, P.L. Senger, J.F. McCutchan & C.A. Landa: Se and GPx distribution in bovine semen and ^{75}Se retention by the tissues of the reproductive tract in the bull. *Biol Reprod* 20, 377-83 (1979)
50. Seligman J, N.S. Kosower, R. Weissenberg & R Shalgi: Thiol-disulfide status of human sperm proteins. *J Reprod Fertil* 101, 435-43 (1994)
51. R Yanagimachi: Mammalian fertilization. In: The Physiology of Reproduction. Eds: Knobil E, Neill J, Ewing LL, Greenwald GS, Market C, Pfaff W, Raven Press, NY (1988)
52. Seligman J, R. Shalgi, Y. Oschry & N.S. Kosower: Sperm analysis by flow cytometry using the fluorescent thiol labelling agent monobromobimane. *Mol Reprod Dev* 29, 276-81 (1991)
53. EM Eddy: The spermatozoon. In: The Physiology of Reproduction. Eds: Knobil E, Neill J, Ewing LL, Greenwald GS, Market C, Pfaff W, Raven Press, NY (1988)
54. Fisher HM & R.J. Aitken. Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. *J Exp Zool* 277, 390-400 (1997)
55. Storey BT: Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol Hum Reprod* 3, 203-13 (1997)
56. Griveau JF, E. Dumont, P. Renard, J.P. Callegari & D. Le Lannou: Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *J Reprod Fertil* 103, 17-26 (1995)
57. Tramer F, F. Rocco, F. Micali, G. Sandri & E. Panfili: Antioxidant systems in rat epididymal spermatozoa. *Biol Reprod* 59, 735-58 (1998)
58. Zini A, E. de Lamirande & C. Gagnon: Reactive oxygen species in semen of infertile patients: levels of superoxide

dismutase- and catalase-like activities in seminal plasma and spermatozoa. Int *J Androl* 16, 183-8 (1993)
59 Gu W & N.B. Hecht: Developmental expression of glutathione peroxidase, catalase and manganese superoxide dismutase mRNAs during spermatogenesis in the mouse. *J Androl* 17, 256-62 (1996)
60. T Mann. In: The biochemistry of semen and the male reproductive tract. Methuen, London (1964)
61. Jeulin C., J.C. Soufir, P. Weber, D. Laval-Martin & R. Calvayrac: Catalase activity in human spermatozoa and seminal plasma. *Gamete Res* 24, 185-96 (1989)
62. Oberley TD, L.W. Oberley, A.F. Slattery, L.J. Lauchner & J.H. Elwell: Immunohystochemical localisation of antioxidant enzymes in adult Syrian hamster tissues and during kidney development. *Am J Physiol* 137,199-214 (1990)
63. Maiorino M, C. Gregolin & F. Ursini: Phospholipid hydroperoxide glutathione peroxidase. *Methos Enzymol* 186, 448-57 (1990)
64. Godeas C, F. Tramer, F. Micali, M.R. Soranzo, G. Sandri & E. Panfili: Distribution and possible novel role of PGHPx in rat epididymal spermatozoa. *Biol Reprod* 57, 1502-8 (1997)
65. Godeas C, F. Tramer, F. Micali, A. Roveri, M. Maiorino, C. Nisii, G. Sandri, E. Panfili: PHGPx in rat testis nuclei is bound to chromatin. *Biochem Mol Med* 59, 118-24 (1996)
66. Roveri A, A. Casasco, M. Maiorino, P. Dalan, A. Calligaro & F. Ursini: PHGPx of rat testis: gonadotropin dependence and immunochemical identification. *J Biol Chem* 267, 6142-6 (1992)
67. Godeas C, G. Sandri & E. Panfili: Distribution of PHGPx in rat testis mitochondria. *Biochim Biophys Acta* 1191, 147-50 (1994)
68. Giannattasio A, M. Girotti, K. Williams, L. Hall & A. Bellastella. Puberty influences expression of PHGPx in rat testis: probable hypophysis regulation of the enzyme in male reproductive tract. *J Endocr Invest* 20, 439-44 (1997)
69. Maiorino M, J.B. Wissing, R. Brigelius-Flohè, F. Calabrese, A. Roveri, P. Steinert, F. Ursini & L. Flohè: Testosterone mediates expression of selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation. *FASEB J* 12, 1359-70 (1998)
70. Imai H, K. Narashima, M. Arai, H. Sakamoto, N. Chiba & Y. Nakagawa: Suppression of leukotriene formation in RBL-2H3 cells that overexpressed PHGPx. *J Biol Chem* 273, 1990-7 (1998)
71. Weitzel F & A. Wendel: Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. *J Biol Chem* 268, 6288-92 (1993)
72. Schnurr K, J. Belkner, F. Ursini, T. Schewe & H. Kuehn: The selenoenzyme PHGPx controls the activity of the 15-lipoxygenase with complex substrates and preserves the specificity in the oxygenation products. *J Biol Chem* 271, 4653-8 (1996)
73. Balhorn R, M. Corzett, J. Mazrimas & B. Watkins: Identification of bull protamines disulfide. *Biochemistry* 30, 175-81 (1991)
74. Brigelius-Flohè R, K.D. Auman, H. Bloeckert, G. Gross, M. Kiess, K.D. Kloeppel, M. Maiorino, A. Roveri, R. Schuckelt, F. Ursini, E. Wingender & L. Flohè: PHGPx. Genomic DNA, cDNA, and deduced aminoacid sequence. *J Biol Chem* 269, 7342-8 (1994)
75. Aitken RJ, E. Gordon, D. Harkiss, J.P. Twigg, P. Milne, Z. Jennings & D.S. Irvine: Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 59, 1037-46 (1998)
76. Ursini F, S. Heim, M. Kiess, M. Maiorino, A. Roveri, J. Wissing & L. Flohè. Dual function of the selenoprotein PHGPx during sperm maturation. *Science* 285, 1393-6 (1999)
77. Li L, A.P. Seddon, A. Meister & MS. Risley: Spermatogenic cell-somatic cell interactions are required for maintenance of spermatogenic cell glutathione. *Biol Reprod* 40, 317-31 (1989)
78. Den Boer PJ, P. Mackenbach & J.A. Grootegoed: Glutathione metabolism in cultured Sertoli Cells and spermatogenic cells from hamster. *J Reprod Fertil* 87, 391-400 (1989)
79. Bauchè F, M.H. Fouchard & B. Jègou: Antioxidant systems in rat testicular cells. *FEBS Lett* 349, 392-6 (1994)
80. Li T-K: The glutathione and thiol content of mammalian spermatozoa and seminal plasma. *Biol Reprod* 12, 641-6 (1975)
81. Storey BT, J.G. Alvarez & K.A. Thompson. Human sperm glutathione reductase activity in situ reveals limitation in the glutathione antioxidant defense system due to supply of NADPH. *Mol Reprod Dev* 49, 400-7 (1998)
82. Kessopoulou E, J.H. Powers, K.K. Sharma, M.J. Pearson, J.M. Russell, I.D. Cooke & C.L.Barrat: A double-blind randomised placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated with male infertility. *Fertil Steril* 64, 825-31 (1995)
83. Suleiman SA, M.E. Ali, Z.M. Zaki, E.M. el-Malik & M.A. Nasr: Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 17, 530-7(1966)
84. Maiorino M, M. Coassin, A. Roveri & F. Ursini: Microsomal lipid peroxidation: effect of Vitamin E and its functional interaction with PHGPx. *Lipids* 12, 721-6 (1989)
85. Godeas C, F. Tramer, G. Sandri & E. Panfili: Rat testis mitochondrial PHGPx does not protect endogenous vitamin E against Fe^{2+}-induced (lipo)peroxidation. *Biochem Mol Med* 58, 221-6 (1966)
86. Williams RM, J.K.Graham & R.H. Hammerstedt. Determination of the capacity of ram epididymal and ejaculated sperm to undergo the acrosomal reaction and penetrate ova. *Biol Reprod* 44, 1080-91 (1991)
87. Daunter B, R. Hill, J. Hennessey & E.V. Mackay: Preliminary report: a possible mechanism for the liquefaction of human seminal plasma and its relationship to spermatozoa motility. *Andrologia* 13, 131-41 (1981)
88. Gagnon C, A. Iwasaki, E. De Lamirande & N. Kowalski. Reactive oxygen species and human spermatozoa. *Ann NY Acad Sci* 637, 436-44 (1991)
89. Gagnon C & A. Iwasaki. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 57, 409-16 (1992)
90. Hinton BT, M.A. Palladino, D. Rudolph & J.C. Labus: The epididymis as protector of maturing spermatozoa. *Reprod Fetil Dev* 7, 731-45 (1995)
91. Zini A & P.N. Schlegel. Identification and characterization of antioxidant enzyme mRNAs in the rat epididymis. *In J Androl* 20, 86-91 (1997)
92. G Poli , E Albano & MU Dianzani: Free radicals: from basic science to medicine. In: Molecular and cell biology updates series, Birkauser Verlag (1993)
93. M Inoue , M Hirota, K Sugi, S Kawamoto, Y Ando, N. Watanabe & Y Morino: Dynamic aspects of glutathione

metabolism and transport during oxidative stress. In: Glutathione Centennial. Molecular Perspectives and Clinical Implications. Eds: Taniguchi N, Higashi T, Sakamoto Y, Meister A, Academic Press, NY (1989)
94. Giblin FJ, J.P. McCready, J.R. Reddan, D.C. Dziedzic & V.N. Reddy: Detoxification of H_2O_2 by cultured rabbit lens epithelial cells: partecipation of the glutathione redox cycle. *Exp Eye Res* 40, 827-40 (1985)
95. A Meister: On the biochemistry of glutathione. In: Glutathione Centennial. Molecular Perspectives and Clinical Implications. Eds: Taniguchi N, Higashi T, Sakamoto Y, Meister A, Academic Press, NY (1989)
96. Aitken RJ & J.S. Clarkson: Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 81, 459-69 (1987)
97. Aitken RJ, J.S. Clarkson & S. Fishel: Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol Reprod* 40, 183-7 (1989)
98. Alvarez JG, J.C. Touchtone, L. Blasco & B.T. Storey: Spontaneous lipid peroxidation and production of superoxide and hydrogen peroxide in human spermatozoa: superoxide dismutase as major protectant against oxygen toxicity. *J Androl* 8, 338-48 (1987)
99. D'Agata R, E. Vicari, M.L. Moncada, G. Sidotti, A.E. Calogero, M.C. Fornito, G. Minacapilli, A. Mongioi & P. Polosa: Generation of reactive oxygen species in subgroups of infertile men. *Int J Androl* 13, 344-51 (1990)
100. Merril AH: Lipid modulator of cell function. *Nutr Rev* 47, 161-9 (1989)
101. Aitken RJ, D. Harkiss & D. Buckingham: Analysis of lipid peroxidation mechanisms in human spermatozoa. *Molecular Reprod Develop* 35, 302-15 (1993)
102. Mazzilli F, T. Rossi, M. Marchesini, C. Ronconi & F. Dondero: Superoxide anion in human semen related to seminal parameters and clinical aspects. *Fertil Steril* 62, 862-8 (1994)
103. De Lamirande E & C. Gagnon: Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. *Fertil Steril* 59,1291-5 (1993)
104. Griveau JF & D. Le Lannou: Effects of antioxidants on human sperm preparation tecniques. *Int J Androl* 17, 225-31 (1994)
105. Griveau JF, P. Renard & D. Le Lannou: An in vitro promoting role for hydrogen peroxide in human sperm capacitation. *Int J Androl* 17, 300-7 (1994)
106. Mortimer D: Sperm preparation techniques and iatrogenic failures of in-vitro fertilization. *Hum Reprod* 2, 173-6 (1991).
107. Gavella M & V. Lipovac: Effect of pentoxifylline on experimentally induced lipid peroxidation in human spermatozoa. *Int J Androl* 17, 308-13 (1994)
108. Gomez E, D.W. Buckingham, J. Brindle, F. Lanzafame, D.S. Irvine & R.J. Aitken: Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl*. 17: 276-87 (1996)

109. Twigg J, N. Fulton, E. Gomez E, D.S. Irvine & R.J. Aitken: Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod* 13:1429-36 (1998)
110. Lanzafame F, M.G. Chapman, A. Guglielmino, C.M. Gearon & R.G. Forman: Pharmacological stimulation of sperm motility. *Hum Reprod* 9, 192-9 (1984)
111. B Halliwell & JMC Gutteridge. In: Free radicals in biology and medicine. Clarendon Press, Oxford (1989)
112. Den Boer PJ, M. Poot, A. Verkerk, R. Jansen, P. Mackenbach, & J.A. Grootegoed: Glutathione-dependent defence mechanisms in isolated round spermatids from the rat. *Int J Androl* 13, 26-38 (1990)
113. Lenzi A, F. Culasso, L. Gandini, F. Lombardo & F. Dondero: Placebo controlled, double blind, cross-over trial of glutathione therapy in male infertility. *Human Reprod* 8, 1657-62 (1993)
114. Lenzi A, F. Lombardo, L. Gandini, F. Culasso & F. Dondero: Glutathione therapy for male infertility. *Arch Androl* 29, 65-8 (1992).
115. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. Cambridge University Press, Cambridge (1999)
116. Aitken RJ & K.M. West: Analysis of the relationship between reactive oxigen species production and leukocyte infiltration in fractions of human semen separated on Percoll gradients. *Int J Androl* 13, 433-51 (1990).
117. L Gandini, A Lenzi, F Lombardo & F Dondero: Glutathione: in vitro effects on human spermatozoa. In: Neuroendocrine and Intragonadal regulation of testicular function. Ed: Marrama P. (1993).
118. Brenner R.R.: Effect of unsaturated fatty acids on membrane structure and enzyme kinetics. *Prog Lip Res* 23, 69-96 (1984)
119. Lenzi A, M. Picardo, L. Gandini & F. Dondero: Lipids of the sperm plasma membrane: from polynsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. *Human Reprod Update* 2, 246-56 (1996)
120. Coniglio JG: Testicular lipids. *Progr Lipid Res* 33, 387-401 (1994)

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