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Review Article

Reactive Oxygen Species and Antioxidant in Seminal Plasma and Their Impact on Male Fertility

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Abstract

Spermatozoa generate reactive oxygen species (ROS) in physiological amounts, which play a role in sperm functions during sperm capacitation, acrosome reaction (AR), and oocyte fusion. In addition, damaged sperm are likely to be the source of ROS. The most important ROS produced by human sperm are hydrogen peroxide, superoxide anion and hydroxyl radicals. Besides, human seminal plasma and sperm possess an antioxidant system to scavenge ROS and prevent ROS related cellular damage. Under normal circumstances, there is an appropriate balance between oxidants and antioxidants. A shift in the levels of ROS towards pro-oxidants in semen can induce oxidative stress (OS) on spermatozoa.

Male infertility is associated with increased ROS and decreased total antioxidant activity in the seminal plasma. ROS induce nuclear DNA strand breaks. Besides, due to a high polyunsaturated fatty acid content human sperm plasma membranes are highly sensitive to ROS induced lipid peroxidation thus decreasing membrane fluidity. This will result in increased lipid peroxidation (LPO), decreased sperm motility, viability, function and ultimately lead to infertility. The protective action of antioxidants against the deleterious effect of ROS on cellular lipids, proteins and DNA has been supported by several scientific studies.

The purpose of the present review is to address the possible relationship between ROS and antioxidants production in seminal plasma, and the role they may play in influencing the outcome of assisted reproductive technology (ART).

Keywords: Reactive Oxygen Species, Total Antioxidants, Infertility, Assisted Reproduction Technology, *In Vitro* Fertilization, Intracytoplasmic Sperm Injection

Introduction

Infinity of infertility

Since the first appearance of humans on earth, infertility has been one of the most controversial medical and social issues. Some civilizations considered it to be a punishment, while others thought of it as an illness. Some blamed it on the female; others could not explain it. This charge of infertility on the female, had serious consequences on her social image and psychological state (1). Additionally, physicians' lack of knowledge of gonadal and sperm function has been a main factor for the female to be considered the one responsible for infertility. It was not until the last decade that our knowledge of the human reproductive system allowed us to determine that a very important parameter of a couple's infertility has been male infertility and more specifically, sperm malfunction (2).

Normal and impaired sperm function

Normal spermatozoa are those that successfully undergo a number of steps necessary for oocyte fertilization. The first step is maturation of the spermatozoa, which is initiated in the male genital tract and concludes with capacitation, the final step of maturation occurring in the female genital tract. Fully mature spermatozoa must swim in the female reproductive system, reach the oocyte, undergo acrosom reaction (AR), penetrate the zona pellucidae and fuse with the oocyte pronucleus to form a zygote (3).

When, after successfully following all the abovementioned steps, the spermatozoa are unable to lead to the natural fertilization of an oocyte, then impaired sperm function is considered. Failed attempts to conceive, after a period of two years, define infertility and the couple must visit an expert to seek diagnosis and possible treatment. The male

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partner undergoes semen analysis and is evaluated according to the World Health Organization reference values. However, there are cases where the semen analysis indicates normal sperm, but the male is still infertile (4). This proves that there are parameters affecting sperm function that cannot be determined by routine semen analysis. Semen analysis includes assessment of seminal volume, spermatozoal motility, density, viability and morphology. Oxidative damage to spermatozoa, induced by excessive production of free radicals or impairment of the natural antioxidant mechanisms, has been identified as such a parameter (5).

Free radicals: origin and oxidative stress
Origin

By definition, a free radical is any chemical compound with one or more unpaired electrons. The free radicals that have been associated with infertility are oxygen and oxygen-derived oxidants, namely, the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO^-) and hydroxyl radicals (OH^-) (6, 7). These oxidants are widely known as reactive oxygen species (ROS) and, due to unpaired electron(s) tend to strongly react with other chemical compounds (8). More specifically, they seek stability by "stealing" electrons from nucleic acids, lipids, and proteins; leading to the damage of cells and disease phenomena (8, 9).

ROS are produced in the body by mitochondria, phagocytes, arachidonate pathways and other physiological processes in which they act as vital signalling molecules. They are products of natural oxygen metabolism and represent approximately 1 to 2% of metabolized oxygen (10). The balance between production and disposal of oxidant molecules is essential for tissue homeostasis. Increased rate of free radical production or decreased rate of removal leads to free radical accumulation and cellular damage (11). Additionally, their production is induced by external factors, such as cigarette smoke, ultraviolet light radiation, and others (7). ROS have been associated with the pathology of numerous diseases such as neurodegenerative diseases (12), vascular disease (13-15), cancer, diabetes, periodontal diseases (16-19) and of course, human infertility (20-28).

ROS and oxidative stress (OS)

The free radicals are a part of an organism's chemical reactions, and are necessary signalling molecules, as well as having a vital role for the maturation processes of several structures. Most free radicals in biology fit within the broader category of ROS, which include not only oxygen–centred radicals such as the superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) or nitric oxide ($NO^{\cdot}$), but also some potentially dangerous non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), peroxynitrite anion ($ONOO^-$), and hypochlorous acid (HOCL) (28). The most common ROS that have a potential implication in reproductive biology include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxyl (ROO^-), and hydroxyl (OH^-) radicals.

The free radical nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$) also appear to play significant roles in reproduction and fertility. Öztezcan et al. (29) indicate that $ONOO^-$ might cause sperm dysfunction through an increase in lipid peroxidation (LPO) and total sulphydryl group depletion. The assumption that free radicals can influence male fertility has received substantial scientific support (30). Many reports have indicated that high levels of ROS are detected in the semen samples of 25% to 40% of infertile men (31). However, small controlled amounts of ROS are vital for spermatozoa to develop normally and be capable of fertilization structures (30, 32, 33).

In addition, de Lamirande et al. and Zini et al. reported that H_2O_2 and O_2^- promote sperm capacitation and AR. H_2O_2 also promotes hyperactivation and oocyte fusion (34, 35). Hydrogen peroxide (H_2O_2) and superoxide anion are of great importance to spermatozoa. They are necessary for controlling the tyrosine phosphorylation events associated with sperm capacitation (32, 36).

ROS production by spermatozoa

ROS are also undoubtedly produced by spermatozoa (37-40), mainly through their mitochondrial system (41), as well as round cells during the spermatogenic process and epithelial cells. In the human ejaculate ROS are mainly produced by leukocytes with marginal amounts produced by spermatozoa (42). The production of ROS in ejaculated spermatozoa is initiated in immature germ cells (39) and continues in the epididymis when the surface of the spermatozoa is remodelled. When the mitochondrial capsule is assembled, chromatin undergoes condensation and motility is acquired for the capacitation of spermatozoa (43, 44). However, O_2^- production by spermatozoa has been questioned (45) on the basis that no free radical signal can be detected by electron paramagnatic resonance (EPR) spectroscopy. Electron leakage from complexes I and II of the mitochondrial transport chain has been proposed as a source of superoxide in male gametes (46). Numerous studies have shown that human sperm exhibit the capacity to generate ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radicals (47, 48). The production of ROS by human sperm is due to a membrane - bound nicotinamide adenine dinucleotide (NADH) oxidase system (47).

Spontaneous generation under aerobic conditions

Spermatozoa generate small amount of O_2^- and NO. which are both tightly related to the cAMP pathway in the control of human sperm capacitation and protein tyrosine phosphorylation (36, 49). Beckman et al., suggested that these two radicals could combine to form peroxy nitrite ($ONOO^-$) (50). Peroxy nitrite is not a free radical because the unpaired electrons of NO and O_2^- have combined to form a new N-O bond in peroxy nitrite, but it is a strong one or two electron oxidant and nitrating agent (51). Nevertheless, generating controlled low amounts of endogenous ROS by spermatozoa play a significant role in inducing sperm capacitation, AR, and acquisition of sperm-fertilizing ability (30, 52).

Retained cytoplasma (RC)

The production of ROS is higher in spermatozoa that are either damaged or retain abnormal cytoplasmic inclusions (39).

Radical production has also been detected in immature germ cells (44). According to Iwasaki and Gagnon (53), the capacity for ROS production is significantly enhanced in abnormal spermatozoa, and those sperm cells with retention of residual cytoplasm. Huszar and Vigue have found that morphological irregularities of sperm are significantly correlated with high creatine kinase (CK) activity (54). ROS production by spermatozoa has been associated with midpiece abnormalities, retained cytoplasm, cytoplasmic droplets and spermatozoa immaturity (48, 55-57).

The primary product of the immature spermatozoa system generating free radicals appears to be the superoxide anion (O_2^-), which secondarily dismutase's to H_2O_2 through the catalytic action of superoxide dismutase (SOD) (58,59). Moreover, the most prevalent ROS, hydrogen peroxide (H_2O_2) is synthesized from O_2 by mammalian spermatozoa (60) by a two-stage reduction of superoxide (O_2^-*) by H+ as the intermediate product (61). The retention of residual cytoplasm in the sperm midpiece after spermination has been associated with excessive production of ROS by spermatozoa (55). The ROS levels are expected to rise at a faster pace and in greater intensity in sperm samples in the presence of cytoplasmic residues. Morphometric analysis of the amount of residual cytoplasm present in the sperm midpiece has revealed significant correlations with the production of ROS (55).

The enzyme gloucose-6-phosphate dehydrogenase (G6PD), which is superfluously exhibited in sperm residual cytoplasm, generates NADN, which in turn stimulates ROS formation (33, 43, 47, 54, 55).

Sperm with cytoplasmic droplets show a higher cellular content of cytoplasmic enzymes, including G6PD. This enzyme is responsible for the flux of glucose through the hexose monophosphate shunt and the associated generation of NADPH. It is theorized that nicotinamide adenine dinucleotide phosphate (NADPH) generated via this system serves as the major source of electrons responsible for the production of O_2^- by human spermatozoa. Therefore, the retention of residual cytoplasm creates a situation in which sufficient substrate would be available to support excessive NADPH–dependent ROS generation (33, 62). Spermatozoa may generate ROS in two ways:

1. NADH-oxidase system at the level of sperm membrane level (37) and
2. NADH-dependent oxido-reductase (diphorase) at the level of the mitochondria (38).

Besides, the biochemical markers of the cytoplasmic space, such as creatine kinase, are positively correlated with the induction of peroxidative damage (55, 56). Huszar and Vigue (56) have found a positive relationship between CK activity and the rate of LPO, as measured by malondialdehyde (MDA) formation in sperm fractions. Hallak et al. have found an inverse relationship between CK levels and sperm morphological forms and suggested that CK levels can be used as a reliable marker for sperm quality and fertilizing potential in subfertile men (63).

Excessive ROS production by immature, morphologically abnormal spermatozoa with cytoplasmic residues such as those confronted in teratozoospermic semen specimens may induce oxidative damage of mature spermatozoa during sperm migration from the seminiferous tubules to the epididymis and may be an important cause of male infertility (64). Immature spermatozoa are a well-characterized source of ROS and a negative correlation between ROS production and semen quality has been documented (39). The excessive generation of ROS by abnormal spermatozoa and by contaminating leukocytes (leukospermia) has been identified as one of the few defined aetiologies for male infertility.

Many reports point out that those biochemical markers of the cytoplasmic space, such as creatine kinase, are positively correlated with the induction of peroxidative damage (55, 56).

Only one third of ROS produced by spermatozoa is released extracellularly (41). In the case of oligozoospermic males whose spermatozoa generate particularly high levels of ROS, the source of cytotoxic oxygen radicals is frequently intracellular (37, 55). However, the main ROS producing sources are immature spermatozoa, especially those with cytoplasmic droplets at the midpiece and leukocytes (65).

ROS production by leukocytes

ROS are produced by leukocytes, which are

present in the male reproductive system and in the ejaculate, as a result of their role in immunological defense against pathogenic germs (26, 66). Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (67). The majority of leukocytes in semen are granulocytes (50-60%), followed by macrophages (20% to 30%) and T lymphocytes (2% to 5%) (4). Leukocytes play an important role in immune surveillance (68, 69) and phagocyte clearance of abnormal sperm (66). However, white blood cells (WBC's) in human semen are also capable of ROS generation (70, 71). Genital tract inflammation and an increased number of leukocytes in the ejaculate have been repeatedly associated with male subfertility and infertility (41, 72-74). Leukocytes are a particularly important source of OS in the ejaculates of patients exhibiting leukocytospermia secondary to infection or as consequence of paraplegia (75). Activated leukocytes can produce 100-fold higher amounts of ROS than nonactivated leukocytes (41). Leukocytes may be activated in response to a variety of stimuli, including inflammation and infection (76).

Whittington and Ford demonstrated that infiltrating leukocytes are the predominant source of ROS production in unspecified sperm preparations (77). Sperm damage from ROS that is produced by leukocytes occurs if seminal leukocyte concentrations are abnormally high such as in leukocytospermia (78), or by removing seminal plasma during sperm preparation for assisted reproduction (72).

Leukocytospermia may induce an alteration in sperm structure by means of excessive ROS production by activated granulocytes. It has been shown that leukocytospermia and excessive ROS levels are associated with an increase in chromatin alterations and DNA damage in sperm, as defined by the sperm chromatin structure assay (79).

In patient samples that generated detectable ROS, the ability of the spermatozoa to retain motility for 24 hours after preparation on a 40/80% Percoll gradient was negatively correlated ($r = -0.310$, $p<0.05$) with basal ROS production (77). Besides, ROS production was also related to the outcome of *in vitro* sperm mucus penetration tests. Unstimulated levels of ROS production showed a significant ($p<0.05$) negative correlation with the number of progressively motile spermatozoa present in the mucus after 15 ($r =-0.379$) and 60 minutes ($r = -0.362$) (77). Wang et al. demonstrated that mitochondrial function was inhibited in the spermatozoa of infertile men and significantly correlated with sperm concentration and the level of ROS production.

ROS and male fertility: physiologica association of ROS

Association with subfertility

Only in the case of excessive ROS production or malfunction of the native antioxidant production mechanisms, do the free oxidants cause problems by putting several tissues under OS (80-83). ROS have been widely associated with both the etiopathogenesis in female infertility (6, 21, 84), as well as with male subfertility and impaired sperm function, but their exact role is still not clear.

It has been more than a decade that the possibility of ROS negatively affecting male infertility has been examined by scientists (30, 82) and there has been an increasing generation of scientific data supporting that view (85). More than 60 years ago MacLeod and Ross et al. described the loss of sperm motility, when the latter is exposed to oxygen at 38°C (86) and 36 years later the first association of OS and impaired sperm function came from the University of Cambridge (87). Since then, numerous scientific publications with very interesting data have come to light, presenting the role of ROS in sperm physiology and morphology (55, 88), and subsequently, the reproductive outcome.

The plasma membrane of spermatozoa contains high amounts of polyunsaturated fatty acids (PUFA), making them highly susceptible to damage by OS (89-91). Furthermore, the concentration of scavenging enzymes, like SOD or glutathione peroxidase (92), in spermatozoa cytoplasm is very low, making the effect of OS more severe (52, 90, 93, 94). Scientific data has been presented to support that ROS negatively affect sperm function by contributing to the occurrence of LPO (47, 91, 95).

Increased production of ROS or impaired action of antioxidant mechanisms can lead to surplus of ROS and hence, OS. However, semen analysis of infertile men showed that increased levels of ROS are a result of over-production of ROS, rather than decreased enrichment of the seminal plasma with antioxidants (96). ROS produced by leukocytes in seminal plasma, sperm itself and other sources and the effect of ROS on male fertility depending on its concentration in seminal plasma despite of their sources of production whether it is pathological or non-pathological source.

The increased production of ROS from immature spermatozoa has been demonstrated in recent studies, indicating a negative correlation between the percentage of normal spermatozoa and levels of ROS production in semen. These results were obtained after calculating ROS production in an ejaculated sperm gradient of immature and mature spermatozoa (based on WHO parameters (4)), presenting high and low ROS production respectively,

and immature germ cells, which also presented with a low production of ROS (39, 97). This excessive production of ROS from immature sperm could cause DNA damage in mature sperm within the male reproductive tract and, hence, account partly for male infertility. It is well documented that there is a negative correlation between defective sperm chromatin structure (DNA-break) and fertility, in *in vitro* fertilization (IVF) cycles (98). However, this condition does not seem mandatory for successful fertilization; as demonstrated by intracytoplasmic sperm injection (ICSI), where normal fertilization and pregnancy rates can be achieved with cells that have not completed spermiogenesis, such as epididymal and testicular spermatozoa (99).
The applied assisted reproduction techniques (ART), as their title indicates, aim at achieving fertilization that is not able to occur naturally. However, research studies have shown that repeated cycles of centrifugation in the process of sperm preparation can induce the production of ROS by spermatozoa. This means that improving the motility of sperm through the routine preparation does not necessary mean that occasional DNA damage does not occur at the same time, endangering the outcome of fertilization (100-103).
Hammadeh et al., showed that ROS and total antioxidants (TAS) concentration in seminal plasma did not differ significantly between the patients undergoing IVF or ICSI therapy, however, negative correlation was found between ROS concentration in seminal plasma and sperm vitality, membrane integrity, sperm density, chromatin condensation, and DNA single stand breaks in both IVF and ICSI groups (104).

Sperm motility and hyperactivation
ROS hydrogen peroxide, in particular, plays a positive physiological role in sperm hyperactivation and capacitation. Low concentration of a NO releasing compound, has been shown to be beneficial to the maintenance of post-thaw human sperm motility and viability (105).
Besides, ROS production has been related to the outcome of *in vitro* sperm mucus penetration tests. ROS showed a significant ($p<0.05$) negative correlation with the number of progressively motile spermatozoa present in the mucus after 15 ($r =-0.379$) and 60 ($r =-0.362$) minutes (77).
Moreover, a highly significant correlation was found between oxidation of sperm DNA and reduced motility [106]. Oxidative stress affects the mean semen parameters (count, motility, morphology) and therfore, asthenozoospermia is probably the best indicator for oxidative stress in a routine semen analysis.

Sperm capacitation
The molecular basis of sperm capacitation is still unclear. Calcium uptake, an increase in cAMP concentration, a rise in intracellular pH, an efflux of cholesterol from sperm plasma membrane (107-108) and tyrosine phosphorylation of specific proteins have been shown to occur during this process (109-110).
Capacitation of spermatozoa may thus occur by different ROS, but it takes place specifically by H_2O_2 following an increase in cAMP, activation of protein kinase A, and downstream tyrosine kinase activation (36).
It has also been demonstrated by many authors (52, 111, 112) that ROS, such as superoxide anion (O_2^{-},) hydrogen peroxidase (H_2O_2), and nitric oxide $NO^{\cdot}$ can induce sperm capacitation *in vitro*. Production of nitric oxide ($NO^{\cdot}$) by spermatozoa has also been reported (51) and may serve as an additional oxidant source. NO has also been suggested to be involved in the capacitation of spermatozoa but only in the presence of H_2O_2 (113).

AR
ROS other than H_2O_2, such as nitric oxide and superoxide anion O_2^{-}, have been shown to promote sperm capacitation and AR (35, 95).
Stimulation of endogenous NADPH-dependent ROS generation in human sperm appears to regulate AR via tyrosine phosphorylation (114).

Sperm egg binding
Aitken et al. demonstrated that low levels of ROS enhance the ability of human spermatozoa to bind to the zona pellucida, an effect that was reversed by the addition of vitamin E. (47).
Treatment of human spermatozoa with low concentrations of NO^{-} releasing compounds like sodium nitroprosside (SNP; $10O^{-7}$ -$10O^{-8}$ M) in the capacitating medium increased the number of spermatozoa bound to the hemizona (115).

Sperm egg fusion
The production of low concentrations of hydrogen peroxidase (H_2O_2 and O_2^{-}) by spermatozoa may have a functional role in the signalling events controlling capacitation and sperm-oocytes fusion (34, 36, 109, 116).
However, poor sperm–oocytes fusion, *in vitro* fertilization experiment (48, 109) and standard IVF (117-120) are related to high ROS production.
Aitken suggests that high failure rate of sperm-oocyte fusion bioassay can be related to increased generation of lipoperoxidase. In a subset of infertile patients sperm were refractory to the second messenger signal generated by Ca ionophore, excessively generated ROS and also exhibited a

high failure rate in sperm-oocytes fusion bioassays (70).

ROS level and fertilization

The redox status of human spermatozoa is likely to affect phosphorylation and adenosine triphosphate (ATP) generation with a profound influence on its fertilizing potential. However, one of the main features of sperm that might profoundly affect fertilization and subsequent development is chromatin structure (121). ROS generation during OS associated with the appearance of damage to the DNA, especially in infertile patients, leads to a high incidence of DNA strand breaks (80, 122). High levels of seminal ROS also impaired the sperm fertilizing capacity by DNA damage and apoptosis (21, 80).

Under normal circumstances, spermatozoa with damaged DNA would not participate in the fertilization process because of collateral peroxidative damage to the sperm plasma membrane (123).

ROS level and cleavage (embryo) quality

The production of ROS may also be involved in bovine embryo development (124). Zorn et al. found that high seminal plasma ROS levels are associated with impaired sperm fertilizing ability and lower pregnancy rates after IVF. In ICSI, a negative association of ROS with embryo development to the blastocyst stage has been observed (125).

ROS level and pregnancy

After IVF, fertilization and pregnancy rates were negatively associated with ROS levels. In ICSI, a negative association of ROS with embryo development to the blastocyst stage has been observed, and significant fewer ICSI derived embryos reached the morula-blastocyst stage on day four (125). In the IVF group (n=26), eleven pregnancies were achieved (42.3%) with a 47.8% implantation rate per embryo transferred. In ICSI patients (n=22), four pregnancies were achieved (18.2%) with a 40.7% implantation rate per embryo transferred as previously described (126).

ROS and sperm DNA damage: pathology of ROS

Several studies have shown that OS caused by ROS production induces damage to the sperm's DNA, even though sperm DNA appears to be more resistant than other cell types such as somatic cells (127). Specifically, sperm DNA is protected by its advanced packaging on the one hand (128) and seminal plasma antioxidants on the other (129). OS-induced DNA fragmentation, which is widely observed in the spermatozoa of infertile men (130, 131), may not directly affect the fertilizing ability of spermatozoa but it directly affects their contribution to normal embryonic development (132) and the mutational load of the embryo (32) (Fig 1). Evidence also supports that OS causes significant damage not only to the nuclear, but also to the mitochondrial DNA of human spermatozoa (133).

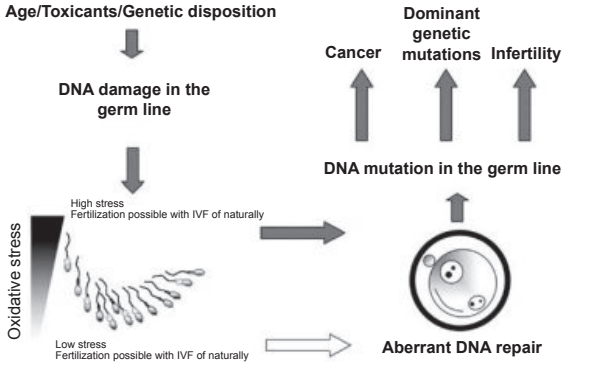

Fig 1: Schematic representation of the possible relationship between OS in the male germ line and abnormalities in the development of the embryo and the health and well-being of the offspring (134).

Along their way from the testis to the oocyte, spermatozoa encounter many factors and conditions that can target their DNA and potentially damage it. Defective packaging of chromatin in the nucleus, apoptosis and ROS are the causes that attract the most scientific interest (135, 136). Sperm DNA damage may also account for the loss of the ability to conceive naturally (137, 138) and for a number of cases of unexplained pregnancy loss (139). As far as ARTs are concerned, their association with DNA damage has been widely discussed and examined in a variety of studies, and extensively reviewed by Agarwal and Allamaneni (140).

OS-induced apoptosis

The programmed death of eukaryotic cells, which occurs without inducing an inflammatory response, is called apoptosis (141). Apoptosis during spermatogenesis has been assessed, discussed and supported in several studies (142-144) and has been associated with male infertility (142, 145, 146). However, research on apoptosis in ejaculated spermatozoa seems to still be in its developing stages, only discussed by a few studies (131, 147, 148), the latest primary article being that of Wang et al. (149). More specifically, the latter group reported a positive correlation between the presence of apoptotic markers, due to OS-induced apoptosis, and spermatozoa DNA damage. OS-induced apoptosis in ejaculated spermatozoa has also been reviewed recently by Agarwal and Said (150).

Role of NADPH and NADPH oxidase activity

ROS are the intermediate steps of oxygen reduc-

tion, namely, O_2^-, H_2O_2 and OH are the products of reduction by one, two or three electrons, respectively. Also, HO_2, the acid of superoxide anion, is another free radical with a major contribution in the destructive LPO process occurring in spermatozoa (89). Exogenous molecules, such as aromatic derivatives and iron complexes with low molecular weight, can activate molecular oxygen by catalyzing electron transfer to it. This can also be done by activating β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which theoretically exists in human spermatozoa and is believed to transfer electrons from NADPH to ground state oxygen, leading to the formation of the superoxide anion radical. The latter is then dismutated to hydrogen peroxide (H_2O_2), which is controlled by the antioxidant glutathione peroxidase. In case the function of the antioxidant is impaired for any reason, the spermatozoa experience hyperoxidation (OS status) (Fig 2) (24). Several other studies have supported the presence of NADPH oxidase-like activity, at the sperm plasma membrane, in human spermatozoa and the role of NADPH in O_2^- production and the subsequent consequences of induced OS on spermatozoa (33, 44, 46, 151, 152). The second ROS-generating system that has been proposed is a sperm diaphorase system, located in the middle piece and integrated into the mitochondrial respiratory system of the spermatozoa (33).

Leukocytospermia and male infertility

Apart from their role as members of the immune-defensive mechanism, leukocytes are also vital for the clearance of defective sperm via phagocytosis (66). Leukocytospermia is a condition which, according to WHO guidelines (4), is determined by the existence of a concentration greater than 1 x 10^6/ml of peroxidase-positive leukocytes in semen and it is encountered in an average of 15% of infertile men (73, 79, 94). Nevertheless, whether or not it is a pathological condition leading to abnormal morphology and impaired sperm function is controversial among scientists.

Specifically, there are studies that have found a negative correlation and other studies that have found a positive correlation of poor sperm quality/function, with increased WBC concentration (leukocytospermia) (74). More specifically, Curi et al. concluded that there is no positive correlation between leukocytospermia and impaired sperm motility (asthenozoospermia) (4, 153). In another study, Tomlinson et al. could not find any association of leukocyte concentration, neither with impaired sperm quality, nor with conception rates (154). Also, no correlation was found between leukocyte counts, sperm density and motility, sperm antibodies and growth of micro-organisms by el-Demiry et al. (67).

On the other hand, Yanushpolsky et al. presented a positive association of increased seminal granulocyte concentrations with abnormal semen parameters of statistical and clinical significance (155). In addition, Arata de Bellabarba et al. showed that increased WBC were a usual phenomenon in the semen of infertile men and associated with semen of poor quality parameters (156). Moreover, Lemkecher et al. supported the positive correlation, but also associated leukocytospermia with increased DNA fragmentation (157). Aitken et al. also presented a positive correlation between leukocytospermia and impaired sperm function, when leukocytes were introduced into the sperm mixture after preparation. The same correlation, when assessed during IVF, was found to be negative (48). In contrast, Vicino et al. showed that increases in leukocyte concentration in semen can affect the results of both IVF and ICSI procedures (158).

Apart from sperm quality, the effect of leukocytospermia on sperm morphology is also a highly controversial issue between researchers. Hence similarly, several studies have presented a positive correlation between increased leukocyte concentration in semen and abnormal sperm morphology. Specifically, Berger et al. showed a positive correlation between morphologically normal sperm and sperm penetration ability, in contrast to morphologically abnormal sperm due to increased leukocyte concentration (159).

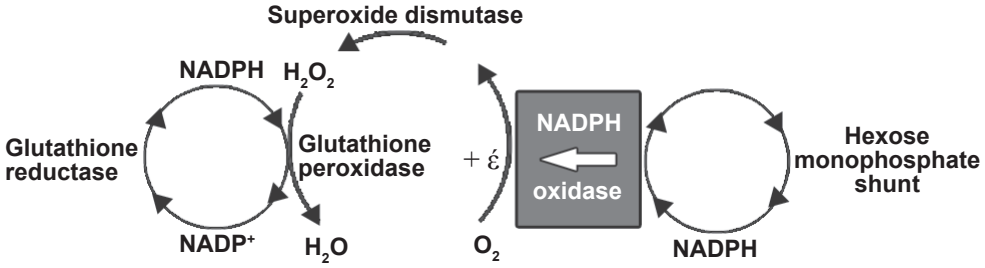

Fig 2: Schematic representation of NADPH oxidase activity (24)

Eggert-Kruse et al. presented a positive correlation between high rates of leukocytes of the round cells with increased morphological abnormality (160) and Yanushpolsky et al. presented statistically significant differences in sperm morphology and leukocyte concentration of double threshold according to the WHO criteria (155). Moreover, Thomas et al. and Menkveld and Kruger presented a positive correlation between WBCs in general and polymorphonuclear granulocytes (PMN) respectively, with morphological defects (161, 162). Finally, two years ago, a primary article by Aziz et al. presented a highly significant negative correlation between leukocytospermia and impaired sperm structural integrity (163).

On the contrary, two more studies presented a statistically insignificant association between increased concentrations of leukocytes and morphologically abnormal sperm (164, 165). To enhance the controversy concerning the role of leukocytes in abnormal sperm morphology, Kiessling et al. and Tomlinson et al. presented a positive correlation between increased leukocyte numbers and morphologically normal sperm, due to leukocytes' physiological function of abnormal-sperm phagocytosis (66, 68).

Lackner et al. (166) suggested that anti-inflammatory medication of male patients with bacterial leukocytospermia improved sperm count and reduced leukocyte concentrations. Furthermore, Oliva and Multigner (167), after using antihistamine-like drug treatment, reported a significant decrease in leukocytes in semen and a significant increase in sperm motility and morphology.

Furthermore, Gambera et al. (168) evaluated the effects of treatment with rofecoxib, a cyclooxygenase-2 inhibitor, on sperm quality and pregnancy rate after intrauterine insemination (IUI) or monitored intercourse. They have shown a significant reduction in leukocyte concentrations, and an improvement of sperm motility and morphology, particularly the presence and shape of the acrosomal complex and tail structure.

Evaluation of the effect of cigarette smoking on antioxidant levels and the presence of leukocytospermia in infertile men showed that lower levels of SOD and catalase were seen in infertile patients compared with fertile donors. SOD was significantly correlated with sperm concentration and negatively correlated with leukocytospermia. In addition, leukocytospermia was inversely correlated with sperm motility. SOD levels were negatively related to cigarette smoking. They concluded that cigarette smoking may impair sperm motility and decrease the antioxidant activity (negative correlation with SOD) in the seminal plasma (169).

Table 1: Some natural antioxidants (Adapted from 186).

Non-enzymatic antioxidant molecules		
Antioxidant Molecule		**Subcellular location**
Ascorbate (vitamin C)		Plastid; apoplast; cytosol; vacuole
β-Carotene		Plastid
Glutathione, reduced (GSH)		Plastid; mitochondrion; cytosol
Polyamines (e.g., putrescine, spermine)		Nucleus; plastid; mitochondrion; cytosol
α-Tocopherol (vitamin E)		Cell and plastid membranes
Zeaxanthin		Chloroplast
Antioxidant enzymes		
Enzyme	**EC number**	**Subcellular location**
Ascorbate peroxidase	1.11.1.11	Plastid stroma and membranes
Proxidases (non-specific)	1.11.1.7	Cytosol; cell wall-bound
Catalase	1.11.1.6	Glyoxysome; peroxisome; cytosol; mitochondria
Superoxide dismutase (SOD)	1.15.1.1	Cytosol (Cu/ZnSOD); plastid (Cu/ZnSOD; FeSOD); mitochondrion (MnSOD); peroxisome
Dehydroascorbate reductase	1.8.5.1	Cytosol; plastid
Glutathione reductase	1.6.4.2	Mitochondrion; cytosol; plastid
Monodehydroascorbate reductase	1.6.5.4	Plastid stroma
Glutathione S-transferases	2.5.1.18	Cytosol; microsomal

Antioxidants and male infertility

Types, role and origin

ROS are necessary for physiological functioning of spermatozoa, but they need to be controlled and their concentrations maintained at a level that is not deleterious to the cells.

This function is carried out by antioxidants (170) (Table 1) present in seminal plasma (26, 171-175). The most common antioxidants, that protect spermatozoa from excess concentrations of ROS and OS-induced damage and altogether represent the total antioxidant capacity (TAC) of seminal plasma are SOD (58, 176), catalase (CAT) (177), the glutathione (GSH) peroxidase system selenium and selenoproteins such as the phospholipids hydroperoxide glutathione peroxidase (PHGPx) and the glutathione reductase system (178, 179), vitamins A, C (180) and E (181), glutathione (182), spermin, thiols, urate (183, 184), albumin, taurine and hypotaurine (171), L-carnitine and zinc. Additionally, the possible protective antioxidant role of vitamins K and D have been reported in literature (185). These antioxidants work together as a wide protective network and many of them become radicals themselves while scavenging oxidants. The rest of the antioxidants in the network ensure that they are regenerated back to their original structures. For example, vitamin C and glutathione regenerate vitamin E. However, this antioxidant protection is not always strong enough to protect from OS. When the volume and distribution of the spermatozoa's cytoplasm is abnormal, antioxidant enzymes are unable to be hosted properly and therefore their defensive chain-breaking function is impaired (42, 87, 90).

A number of reports have discussed the possible origin of antioxidants in semen, but our knowledge is still limited. Some studies supported the testicular origin of semen antioxidants, while others have presented evidence that the source of antioxidant activity is post-testicular. More specifically, in 1994, Bauche et al. presented evidence that SOD is of testicular origin, protecting it from deleterious ROS concentrations (187). In 1998, research by Yeung et al. confirmed that the source of antioxidants is post-testicular, most probably the prostate and seminal vesicles (188). Four years later, Zini et al. improved that report, showing that antioxidants are post-testicular products and their probable role is the protection of ejaculated spermatozoa from OS such as that which occurs in the female reproductive tract (175). However, during spermatogenesis and epididymal storage, sperm must rely on epididymal/testicular antioxidants and their own intrinsic antioxidant capacity for protection. Testicular biopsies from men with varicocele-have shown an increase in oxidative DNA damage within spermatogonia and spermatocytes (189).

Association with subfertility: a remedy?

ROS increase or antioxidant deficit have been associated with a number of pathological conditions by several scientific studies. The protective action of antioxidants against the deleterious effect of ROS on cellular lipids, proteins and DNA has been supported by several scientific studies (173). In this review, we separated the publications into three groups; namely, studies discussing natural (enzymatic), synthetic (non-enzymatic) and both, respectively.

Antioxidants, in general, are free radical scavengers that suppress the formation of ROS and/or oppose their action. There are many antioxidants in seminal fluid which can be divided into two groups: enzymatic like such as SOD, catalase (96), and glutathione peroxidise, (178) and nonenzymatic antioxidants such as ascorbate, urate, alpha-tocopherol, pyruvate, glutathione, taurine, and hypotaurine (93). Antioxidants that are present in the seminal fluid compensate for the deficiency of cytoplasmic enzyme in the spermatozoa (190). Spermatozoa themselves posses high concentrations of thiol groups, as well as smaller amounts of ascorbic acid, alpha-tocopherol, uric acid and GSH (179, 191, 192).

ROS production and TAC can be used as a marker of OS in seminal fluid and has been correlated with male infertility. Infertile men with male factor or idiopathic diagnoses had significantly lower ROS-TAC scores than controls (85). Said et al. suggested that abnormal sperm morphology combined with elevated ROS production may serve as a useful indicator of potential damage to sperm DNA (193).

Lisak et al. examined the effect of oxidation of protein and lipids by a thermochemiluminescence (TCL) analyzer and showed that thermochemiluminescence indices in seminal plasma closely correlate with sperm characteristics among patients with sperm disturbances (194).

Morphologically abnormal and immature spermatozoa that retain cytoplasmic residues in the midpiece can be separated by a double-density gradient procedure (39, 97, 195). Recently, a study conducted by Mendiola et al. (196) to compare dietary habits in normospermic and oligoasthenoteratospermic patients found that frequent intake of lipophilic foods like meat products or milk may negatively affect semen quality in humans, whereas some fruits or vegetables may maintain or improve semen quality. In another study of the same group, to compare the effect of specific nutrient intake, they found that a low intake of antioxidant nutrients was associated with a poor semen quality

in men that attended an infertility clinic (197).

Enzymatic antioxidants

Human sperm contains the enzyme system comprising glutathione peroxidase (GPX), glutathione reductase (GRD); and their substrates, glutathione (GSH) and glutathione disulfide (GSSG) (59, 178, 179). This system functions as a defence against LPO in human sperm by reducing formed lipid hydroperoxides, which are very reactive and damaging to the plasma membrane; and to hydroxylipids, which are essentially inert (198).

SOD, catalase, and glutathione peroxidase are antioxidants that convert superoxide (O_2) and peroxide (H_2O_2) radicals to form O_2 and H_2O.

Superoxide dismutase (SOD)

One family of antioxidants used by organisms as a defence mechanism against oxidative damage, is located in the family of SOD (Fig 3) (199-201). One of the members of this family, namely, extracellular superoxide dismutase (EC-SOD), is a vital part of the defense against ROS-mediated tissue damage, and is located in the extracellular matrix of tissues, hence, the name EC-SOD (202, 203). SOD acts to scavenge the superoxide anion, which is produced by a one-electron reduction of an oxygen molecule and initiates a radical chain reaction.

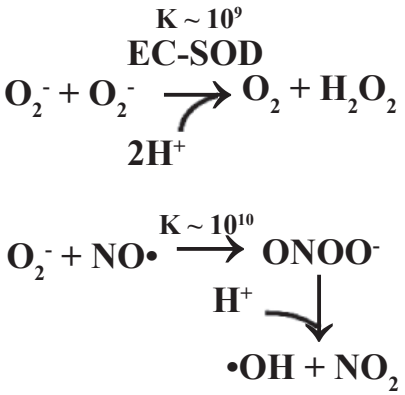

Fig 3: The enzymatic dismutation of superoxide anion by SOD, such as EC-SOD. In addition, the reaction of superoxide with nitric oxide to produce peroxynitrite anion is depicted along with the products of peroxynitrite decay (Adapted from 204).

In addition, the reaction of superoxide with nitric oxide to produce peroxynitrite anion is depicted along with the products of peroxynitrite decay (adapted from 204).

In 2000, Potts et al. reported that seminal plasma, whose role is to protect spermatozoa from DNA damage and LPO, contains antioxidants (205). Three years later, Lamond et al. presented evidence that adding SOD to IVF media would protect sperm chromatin from breakdown (206). Earlier, in 1992, Nonogaki et al. had shown that adding SOD to culture medium protected sperm viability and the embryo development *in vivo* and *in vitro* (207).

The SOD antilipoperoxidative defence system in human sperm relies almost entirely on the activity of a single enzyme, the Cu/ZN isoform (208).

SOD protects against spontaneous O2 toxicity and lipid peroxidase (209). SOD and catalase also remove (O_2^-) generated by NADH-oxidase in neutrophils and can play an important role in protecting spermatozoa during genitourinary inflammation (210).

The steady-state concentration of superoxide is under the control of extracellular SOD, the inhibition of which (by copper chelating) leads to the rapid disappearance of thiols (211). A pivotal role of SOD in protection of testicular cells against heat stress–induced apoptosis has been demonstrated in vivo and in vitro (212, 213).

Murawski et al.(214) showed a positive correlation between SOD activity in seminal plasma and semen quality parameters, sperm concentration and overall motility. SOD activity in oligoasthenozoospermic patients was significantly lower compared to the activity found in normospermic men. They came to the conclusion that decreased seminal plasma scavenger antioxidant capacity, particularly in the form of low SOD activity, can be responsible for male infertility.

Catalase

Catalase is a well known antioxidant enzyme whose localization is limited to peroxisome. Catalase activity has also been determined in human spermatozoa and the seminal plasma of fertile and infertile males (177). However, a difference in the seminal catalase activity of asthenozoospermic and oligo-asthenozoospermic patients with hyperviscosity has been shown (215).

Tavilani et al. (216) found an inverse correlation between activities of CAT and SOD in seminal plasma with an MDA content of spermatozoa from normozoospermic samples. However, such correlations observed between activities of CAT and GPX of seminal plasma with MDA content of spermatozoa of asthenozoospermic patients. Furthermore, they found positive correlations between total activities of CAT, SOD and GPX with total content of MDA in seminal plasma from normozoospermic samples. In asthenozoospermic samples, there were no such significant correlations. They suspected that under pathological conditions (e.g. asthenozoospermia) the activity of seminal antioxidant enzymes can not protect spermatozoa and may cause an increase of lipid peroxidation from spermatozoa.

Protein and SH group (SH-group)
A sulfhydryl group (SH-group) plays an important role in sperm metabolism and antioxidative defence. Nakamura et al. demonstrated that seminal plasma SOD, catalyse, glutathione peroxidase and sulfhydryl group levels are significantly lower in infertile patients than those in controls, suggesting their relationship to male infertility (217).

Glutathione
Reduced glutathione is also part of the antioxidant system in the seminal fluid. Glutathione is a tripeptidyl molecule and present in either the reduced gluathione (GSH) or oxidized form gluathione (GSSG) by forming a disulfide bond between two molecules.
Lenzi et al. found that glutathione injection (600 mg/day, i.m.) for two months significantly improved the morphology and motility patterns of spermatozoa. Even the *in vitro* use of glutathione treatment increased the forward motility and migration from the pellet of spermatozoa obtained from leukospermia samples (218). Parinaud et al. showed *in vitro* enhancement of sperm motility using a migration salt solution containing glucose and glutathione as an antioxidant (219).
A highly significant decrease of mean levels of antioxidants in seminal plasma (glutathione, ascorbic acid and total antioxidant status) were found in oligozoospermic and azoospermic cases compared to normozoospermic controls. Whereas, malondialdehyde level was significantly elevated in oligozoospermic and azoospermic men (220-222).

L,N-acetyl-cysteine
Glutathione (γ-glutamyl-cystenyl-glycine) is a natural, highly effective reducing agent. L, N-acetylcysteine is a potential regulator of germ-cell death and is a well established inhibitor of physiological cell death in several systems. N-acetyl-L-cysteine, a reducing substance, has been shown to improve sperm motility in vitro together with a decrease of ROS levels in infertile patients with a high seminal level of ROS. L, N-acetyl-cysteine (0.1, 1 and 5 mg/ml) has a dose dependent effect in reducing ROS levels; the reduction was greater in patients with high levels of ROS than in those with low levels (223). L, N-acetyl-cysteine and/or a mixture of essential fatty acids and natural vitamins A and E reduced levels of 8-hydroxy-2`-deoxyguanosine (8-OH-dG), are considered as a markers of OS-induced sperm DNA damage (140).
N, L acetyl-L-cysteine, when given in concentrations of 125, 100, 50 and 25 mmol/L, suppressed germ cell death in a dose-dependent manner, however, more research is needed to validate its efficacy in vivo (224).

Glutathione peroxidase
Glutathione peroxidase and catalase activities are of prostatic and multi-glandular origin, respectively (188).
Glutathione peroxidase, a selenium-containing antioxidant enzyme with glutathione as the electron donor, removes peroxyl radical from various peroxides including H_2O_2 to improve sperm motility. Minor alterations in sperm membranes in selected cases of dyspermia can be reversed by glutathione (GSH) therapy (225).
The presence of glutathione may prevent the accumulation of peroxynitrite ($ONOO^-$) to toxic levels and may convert $ONOO^-$ to secondary products with protective properties (226).
Low levels of NADH and glutathione, as a result of the increased activity of glutathione peroxidase to remove metabolites of membrane LPO will further affect cellular Ca^{+2} homeostasis (178).
The selenium-containing enzyme glutathione peroxidase destroys peroxides before they can damage the cell membrane and interacts synergistically with vitamin E (227).
Glutathione peroxidase has been measured in seminal plasma and correlated with male infertility (228). Yeung et al. studied the GPX, GSH reductase, and SOD activities of normozoospermic patients and patients undergoing *in vitro* fertilization treatment and suggested that the origin of the GPX, GSH, reductase, SOD is neither testicular nor epididymal (188).

Glutathione reductase
Glutathione peroxidase is one of the principal antioxidant defence enzymes in human spermatozoa, but it requires oxidized glutathione to be reduced by glutathione reductase using NADPH generated in the pentose phosphate pathway. Glutathione reductase regenerates reduced GSH from its oxidized form (GSSG). GSH has a likely role in sperm nucleus decondensation. Also, intracellular GSH concentrations found to be lower when sperm morphology is severely impaired in both fertile and infertile males (229).
Williams and Ford (230), showed that the pentose phosphate pathway in human spermatozoa can respond dynamically to oxidative stress and that inhibiting glutathione reductase impairs the ability of sperm to resist lipid peroxidation. They concluded that the glutathione peroxidase-glutathione reductase-pentose phosphate pathway system is functional and provides an effective antioxidant defence in normal human spermatozoa.

Alpha-glutamyl transpeptidase
Alpha-glutamyl transpeptidase is present in the midpiece and acrosomal region of spermatozoa and it may further regulate the GSH content of oocytes at the time of sperm penetration (225).

Non-enzymatic antioxidants
Besides the enzymatic antioxidant system, there are numerous non-enzymatic low molecular mass antioxidants which are believed to be even more important scavengers than high molecular mass compounds (96).

Nitric oxide
NO is synthesized from L-arginine by a family of enzymes known as the NOS. NO is a molecule of great biological significance and has long been considered to play an important role in sperm physiology (231).
NO can act as a free radical scavenger inactivating (59, 232) and even inhibiting production of superoxide anion ($O_2^{\cdot -}$) (233) which cause LPO, a process which leads to the functional impairment of spermatozoa (234). NO is produced directly by spermatozoa and constitutive NOS is present in two isoforms similar to those present in both endothelial (ecNOS) and in brain (bNOS) cells. Besides, spermatozoa from normozoospermic samples appear to have greater amounts of NOS and higher amounts of NO production than those from asthenozoospermic samples (235).

Albumin
Human serum albumin (HSA) present in culture media protects antioxidants. HSA has a cysteine residue in position 34 which is not involved in a disulfide bond and may exist in a different oxidative state: as a fully reduced sulfhydryl group, as a mixed disulfide with cysteine, glutathione or homocysteine, for example, or in a higher oxidative state like sulfonic acid. Serum albumin is discussed as a marker for systemic OS (236).
Albumin is reported to exhibit an excellent ability to sustain sperm motility (152).

Zinc
Zinc is an element whose importance in the biological systems is undisputed. Its importance can be understood by considering that when it is deficient, severe pathological consequences occur, such as acrodermatitis enteropathica, a rare autosomal-recessive inheritable disease (237). As far as reproduction is concerned, this element has been shown to be highly important for conception, successful implantation and pregnancy outcome (238, 239). Zinc is present in high concentrations in the seminal fluid and there is evidence that it may act *in vivo* as a scavenger of excessive O_2^{-} production by defective spermatozoa and/or leukocytes in semen after ejaculation (240).
There is evidence that zinc plays a vital role in the physiology of spermatozoa and spermatogenesis. Specifically, Bedwal and Bahuguna reported that this element decreases testicular weight and causes shrinkage of seminiferous tubules (241). Its potential role in sperm production, viability and prevention of spermatozoa degradation and sperm membrane stabilization has also been supported (242). After ejaculation, the present abnormal spermatozoa are sources of oxidants, namely, superoxide anions, which bind with zinc and reduce its concentration in seminal plasma. Thus, zinc is considered to be a vital antioxidant, guarding normal spermatozoa against superoxide anion-induced OS (41, 225). Chia et al. supported the research(?) that zinc concentration in seminal plasma is significantly correlated with sperm density, motility and viability (243).
The biological function of zinc and the characteristic features of zinc deficiency have been reviewed (244, 245).
Zinc is vital for spermatogenesis and for the development of primary and secondary sexual characteristics (246). Experimental zinc deficiency in humans leads reversibly to reduced sperm count combined with reduced serum testosterone (247). There is a link between zinc deficiency and oligospermia (248).
The total zinc content of mammalian semen is high, 800-3000 μm/g of dry weight, and it has been demonstrated that zinc deficiency induces atrophy of the seminiferous tubules and causes failure of spermatogenesis in rats (249). It has been suggested that the zinc ion exchange takes place between the epididymal epithelium and sperm cells as they pass along the epididymal duct and it might be significant for the maturation process of rat sperm cells during their passage through the epididymis (250, 251).
Ali et al. (252) showed a significant difference in serum and seminal zinc levels in normospermic, oligospermic ($p<0.05$) and azoospermic ($p<0.005$) subjects. Levels of zinc in seminal plasma is correlated positively with sperm count and correlated negatively with sperm motility in both normospermic and oligospermic group, whereas levels of seminal plasma zinc showed negative correlations with semen volume, pH and leucocytes concentration in all three groups (normospermic, oligospermic and azoospermic).
They concluded that zinc may contribute to fertility through its significant effects on various semen parameters and may help in the investigation and treatment of infertile males.

Others studies demonstrated that both folate and zinc have antioxidant properties that counteract ROS. Folate, zinc, ROS and thiols affect apoptosis, which is important for sperm release, regulation of follicle atresia, degeneration of the corpus luteum and endometrial shedding (253).
However, no consistent associations were found between antioxidant or zinc intakes and sperm aneuploidy (254).

Vitamins C/E

The most important antioxidants in seminal fluid seem to be vitamins C and E (180, 181). The concentration of vitamin C in seminal plasma is ten times greater than in blood plasma (364 vs. 40 μmol/L) (191). During the nonenzymatic recycling pathway for vitamin E regeneration that occurs in membranes, vitamin C reduces chromanoxyl radicals to recycle vitamin E and eventually being itself consumed by the process (255). Vitamin C intake may reduce DNA strand breaks (256) in human lymphocytes. Dietary supplementation of vitamin C protects human sperm from endogenous oxidative DNA damage (257). Hughes et al. found that *in vitro* treatment of sperm with antioxidants (300 and 600 μM ascorbic acid; 3 and 60 μM alpha-tocopherol, and 400 μM urate) reduce the magnitude of DNA damage as measured by the comet assay (258, 259).
Vitamin E is a term that encompasses a group of potent, lipid-soluble, chain-breaking antioxidants. Structural analyses have revealed that molecules with vitamin E antioxidant activity include four tocopherols (αβγδ) and four tocotrienols (αβγδ) (260). Vitamin E is known to readily reduce alkyl peroxy radicals of unsaturated lipids (261). It is a major chain-breaking antioxidant in the sperm membranes and it appears to have a dose-dependent protective effect (262).
Vitamin E is proven to be effective in preventing LPO and other radical-driven oxidative events (263). Vitamin E functions as a chain-breaking antioxidant that prevents the propagation of the free radical reaction (264, 265). Vitamin E prevents the loss of spermatogenesis in males and failure to retain the zygote in female rats (266). Many investigators have found a reduction in the concentration of oxidized DNA (8-hydroxy-2 deoxyguanosine) in sperm after antioxidant supplementation (130, 267).
Vitamin E inhibits LPO in membranes by scavenging peroxyl (RO.) and alkoxyl (ROO.) radicals. However, the ability of alpha-tocopherol to maintain a steady-state rate of peroxyl radical reduction in the plasma membrane depends on the recycling of α-tocopherol by external reducing agents such as ascorbate or thiols (227).
Semen vitamin E levels were not increased after three months of daily treatment with 400 mg vitamin E plus 500 mg vitamin C, although there was a marked increase in serum concentration. Ascorbic acid (vitamin C) is an important water-soluble antioxidant that reduces sulfhydryls, scavenger's free radicals, and protects against endogenous oxidative DNA damage (257).
Small doses of vitamin C (200 mg), have been shown to increase the seminal level of ascorbate in smokers from 5.6 to 13.1 mg/dl, which is similar to the level achieved (16.1 mg/dl) after 1000 mg of vitamin C (268). In infertile patients with a high level of oxidative DNA damage in spermatozoa, even the combination of vitamin C and E with glutathione induced only a slight increase in sperm concentration (130). Concurrent administration of vitamin C (350 mg/d) and E (250 mg) *in vivo* were not able to prevent sperm DNA damage occurring after ejaculation (258). Vitamin C may become a pro-oxidant when free transition metals are present (92, 269).
Oral supplementation with vitamin C has been shown to improve sperm counts, motility, and morphology in infertile patients (270).
Seminal ascorbic acid level was significantly lower in patients with leuckospermia than patients with normal semen parameters. Interestingly, a significantly greater percentage of men with abnormal DNA fragmentation index (DFI) were observed in the patients with low levels of seminal ascorbic acid compared to those with normal or high levels of ascorbic acid (59% vs. 33%, $p < 0.05$). Therefore, they came to the conclusion that insufficient seminal ascorbic acid was frequently associated with sperm DNA damage (222).
Vitamins C and E both have antioxidant properties and have been shown to be implicated in metabolic pathways of free radical scavenging and OS protection (271, 272). Vitamin E specifically interacts with Se-containing glutathione peroxidase to prevent the oxidative breakdown of tissue membranes (273) and has been shown to improve sperm motility when supplemented orally (274), as well as the in vitro function of spermatozoa (275). In 1996, Geva et al. showed that treatment with vitamin E can protect from LPO and the consequent changes to the sperm (276). A number of clinical studies have concluded that supplementation of vitamins C and E in specific combinations, results in protecting OS-induced sperm DNA damage. More specifically, vitamins cause a decrease in 8-OH-dG levels, which is thought to be a marker of sperm DNA damage induced by OS (140). Moreover, evidence supports that adding vitamins C and E in the medium during sperm preparation reduces hydrogen peroxide and, hence, protects

against OS (19). Supplementation of ascorbic acid (vitamin C) during an earlier study resulted in significantly improved sperm quality (268).
Nouri et al. (277) studied the relationship between levels of LPO (malonadialdyhide, MDA) and vitamins E and C concentrations in the sperm and seminal plasma of asthenoteratozoospermic (AOT) and normozoospermic men and their effect on semen parameters. They found that the level of vitamins E and C in seminal plasma of normozoospermic were significantly higher in asthenoteratozoospermic males, whereas MAD levels were significantly higher in AOT in comparison to normozoospermic males.

Urate
Ascorbate, urate and protein sulfhydryls are the major antioxidants present in seminal plasma, while GSH is practically undetectable (188).
The proposed protective function of uric acid against free radicals in human blood (278) has not been sufficiently investigated in seminal plasma. Very little data could be found in the literature on its presence in seminal plasma (279) and on its antioxidative defence properties (280). Gavella et al. found changes in total seminal plasma antioxidative capacity chiefly due to ascorbate and urate (183). Orally supplemented ascorbic acid is rapidly distributed in all body tissue with the highest concentration in seminal vesicles (281).

Selenium
Selenium, in the form of selenocysteine, functions as the catalytic centre in the active sites of at least nine human enzymes, including four glutathione peroxidase antioxidant enzymes (282-284). It is well known that selenium is involved in the male reproductive process. In mature rats the testicular content of selenium increases greatly at the beginning of spermatogenesis (285). Selenium is also required for normal testicular development and spermatogenesis in rats (286). Rats with low selenium levels produced sperm with impaired motility and characteristic mid-piece damage (287, 288) indicating that selenium is necessary for normal sperm development.
The specific role of selenium in spermatogenesis appears to be related to the phospholipid hydroperoxide glutathione peroxidase, which is expressed depending on the developmental state of spermatids and seems to be converted into a structural component in the mid-piece of mature spermatozoa (289). Selenium deficiency is associated with impaired sperm motility, structural alteration of the mid-piece, and loss of the flagellum (290). In mature spermatozoa, selenium is largely restricted to the mitochondrial capsule, a keratin-like matrix that embeds the helix of mitochondria in the sperm mid-piece (291).
Phospholipids hydroperoxide glutathione peroxidase is expressed at higher levels in rat testes than in any other tissue (292). Deficiencies of selenium or glutathione can lead to instability of the mid–piece of spermatozoa, resulting in defective motility (293, 294).
Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a unique intracellular enzyme. Its uniqueness lies in the fact that it has the ability to reduce intracellular membrane phospholipid hydroperoxides, hence, its name (295). The highest activity of PHGPx has so far been measured in the testis, with a significant difference from that of other tissues, such as the brain and liver (296, 297). Due to the fact that sperm cells have high numbers of polyunsaturated fatty acids, they depend on PHGPx (together with other scavenging systems) to protect them from OS and LPO (298) which is the reason its concentration in spermatozoa is relatively high (295).
In 2005, Greco et al. presented an interesting study, supporting the antioxidant role of vitamins C and E. More specifically, they detected a reduction in ejaculated spermatozoa DNA fragmentation after treatment with a combination of the two vitamins. The interesting aspect of this study was that it was the first study to present the direct effect of antioxidant treatment on sperm DNA integrity *in vivo* (299). Glutathione peroxidase has been shown to maintain sperm motility through scavenging of peroxyl radicals ($ROO^{\cdot}$) (300) and also protect sperm from LPO (301). Additionally, GSH has been shown to be a vital antioxidant mechanism maintaining normal sperm motility (62, 174) and the ability of sperm to undergo AR (62, 174). In an earlier study, Lenzi et al. presented that glutathione therapy for two months had a statistically significant positive effect on sperm motility and morphology (302).

Treatments with antioxidants
According to a study carried out on patients with high levels of DNA damage or LPO and with a history of recurrent pregnancy loss, it was shown that increasing the intake of antioxidant could result in an improvement in pregnancy outcomes(303). After 90 days of treatment with the immune-modulating and anti-oxidants (beta-glucan, papaya, lactoferrin, and vitamins C and E) in patients with asthenoteratozoospermia associated with leukocytosis, Piomboni et al. (304) found a significant reduction in seminal fluid leukocyte concentrations (2.2 ± 0.9 vs. 0.9 ± 0.2) and consequently an increase in the percentage of morphologically normal sperm ($17.0 \pm 5.2\%$ vs. $29.8 \pm 6.5\%$), to-

tal progressive motility (19.0 ± 7.8 vs. 34.8 ± 6.8), and chromatin integrity. In a prospective study performed on men with persistent oligospermia (5–20 million/ml) a statistically significant (p= 0.009) increase in sperm count after antioxidant therapy was recorded (305). In addition, univariate logistic regression analysis showed that men treated with antioxidant therapy presented with the probability of having a normal sperm count that was 20-fold (OR = 20.1; CI 95% = 1.05–43.2; p = 0.014) higher than a untreated men. The authors (305) are of the opinion that antioxidant therapy based on a combination of N-acetylcysteine (NAC) and micronutrient (vitamins- minerals) supplementation can be helpful in improving the sperm count at least in a subset of oligospermic males.

Moreover, studying the effect of zinc therapy in asthenozoospermic men showed that zinc therapy alone, in combination with vitamin E or with vitamin E and C was associated with an increase of sperm parameters and decrease of OS, sperm apoptosis and sperm DNA fragmentation index (DFI) (306).

Sperm cryopreservation extender supplemented with melatonin, pyruvate and taurine as antioxidants can improve semen motility after a freeze thawing procedure (307).

Conclusion

ROS have been associated with the pathology of numerous diseases. Small controlled amounts of ROS are vital for spermatozoa to develop into normal spermatozoa capable of fertilization structures. Hydrogen peroxide, H_2O_2, and O_2^- promote sperm capacitation and AR In addition, hydrogen peroxide (H_2O_2) promotes hyperactivation and oocyte fusion. Besides, hydrogen peroxide and superoxide anion are of great importance to spermatozoa, and are necessary for controlling the tyrosine phosphorylation events associated with sperm capacitation.. Additionally, ROS are produced by leukocytes present in the male reproductive system and in the ejaculate, as a result of their role in the immunological defense against pathogenic germs. Only in case of excessive production of ROS or malfunction of the native antioxidant-production mechanisms, do the free oxidants cause problems by putting tissues under OS.

The function of controlling excessive ROS production is carried out by antioxidants that are present in the seminal plasma. A number of reports have discussed the possible origin of antioxidants in semen but our knowledge on that is still limited. Some of the studies supported the testicular origin of semen antioxidants, while others have presented evidence that the source of antioxidant activity is post-testicular.

A review of the literature shows that the evidence about the scavenging role of antioxidants and sperm protection is controversial. Some researchers have presented evidence of a defensive role of antioxidants, others have found that both deficient and excessive concentrations of antioxidants are deleterious for the sperm and others have shown the effect of antioxidants on some sperm parameters but not all of them. The existing scientific data concerning the scavenging ability of organic and inorganic antioxidants is very interesting but their actual origin and their mechanism of action in the male reproductive system and on ejaculated spermatozoa warrant further studies.

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