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During the last several decades, the quality of human sperm and its fertility potential have decreased dramatically (Dikshit et al., 1987; Menchini - Fabris et al., 1996). This may suggest that the quality of semen has deteriorated partly due to the effects of increasing toxic factors in the environment (Nelson and Bunge, 1974; Leto and Frensilli, 1981; Menkveld et al., 1986). The effect of pesticides, lead poisoning and radiation are some of the most well-known of environmental factors. There has however been a tendency to overlook the mutagenic consequences of cigarette smoke condensates and their possible effects on germ cell maturation and reproduction (Kulikauskas et al., 1985). In man, these effects may be manifested, among other parameters, by abnormalities in the quality of sperm (Wong et al., 2000). Although tobacco (Nicotiana tabacum) smoking is widely recognized as a major health hazard and a cause of preventable mortality, consumption of tobacco remains prevalent in human society (Gorrod & Wahren, 1993). Approximately, one-third of the world’s population (>15 years) smokes regularly (World health organization, 1997). Cigarette smoking is known to be detrimental to health and fertility potential (Stillman et al., 1986; Zenzes, 2000). Epidemiological studies from the general population of reproductive age have provided evidence of dose-related effects of smoking, resulting in a conception delay of ~2 months (Bolumar et al., 1996) and an advance in the age of menopause by ~2 years (Midgette and Baron, 1990). This suggests that certain components in cigarette smoke interact, directly or indirectly, with the gamete cells affecting their function and viability. The reproductive system is complex in nature and therefore, many sites from the hypothalamic-pituitary axis to the germinal cells, can be vulnerable to the disruption of reproduction (Zenzes, 2000).

Cigarette smoke contains several substances, including nicotine, carbon monoxide, cadmium, benzo (a) pyrene, naphthalene, radioactive polonium, and...
other carcinogens and mutagens (Stedman, 1986; Gorrod and Wahren, 1993; Zenzes, 2000) that are harmful to germ cells. Numerous investigations have been conducted on the relationship between smoking and male infertility (Calzada et al., 1992; Pekarsky et al., 1995; Gandini et al., 1997; Merino et al., 1998; Wong et al., 2000), however the molecules mechanisms are not well understood in most of the cases.

In habitual heavy smokers, long-term exposure to tobacco smoke whose by-products move across the blood-testis barrier and exist at high concentrations in the testes may adversely affect the sperm function (Stedman, 1986). In the United States of America, approximately 36% of men of reproductive age smoke some types of tobacco products, not including those exposed to passive or sidestream smoke (Taymor, 1990). However, several investigators have reported little or no effect of smoking on sperm quality (Godfrey, 1981; Rodriguez-Rigau et al., 1982; Hodias et al., 1985; Vogt et al., 1986; Dunphy et al., 1991; Wong et al., 2000). While others have found a significant correlation between smoking and the impairment of semen parameters, such as decrease of sperm motility or an increase in abnormal sperm morphology and lowered sperm density (Evans et al., 1981; Shaarawy and Mahmoud, 1982; Vine et al., 1994; Merino et al., 1998 & Zenzes, 2000). Also, exposure of rats to cigarette smoke produced accumulation of lipids in the tissues causing cardiovascular complications (Latha et al., 1993). Peroxidative damage was reported to be a major contributing factor for such complications (Helen and Vijayammal, 1996; Steinberg and Chait, 1998).

For women, there is evidence that cigarette smoking imparts an elevated risk for a wide variety of abnormal reproductive outcomes which include infertility, spontaneous abortion, stillbirth and neonatal death, low birth weight, congenital malformations, and other abnormal reproductive events (Abel, 1980; Seidman et al., 1990). Moreover, paternal smoking during preconception period has been associated with childhood brain cancer in the offspring (Ji et al., 1997). However, the mechanism by which paternal smoking
may be linked to detrimental effects on offspring are poorly understood. Smoking also may induce higher proportions of genetically defective sperm, given the observations that cigarette smoke contains more than 30 chemical agents known to be mutagens, clastogens, aneugens or carcinogens in model systems (Lofroth, 1989; Claxton et al., 1989). Smokers have been shown to have increased endogenous DNA strand breaks in sperm (Potts et al., 1999), increased fractions of peripheral blood lymphocytes with mutations in the hypoxanthine phosphoribosyltransferase (HPRT) gene (Jones et al., 1993), and chromosomal aberrations (Tucker and Moore, 1996). Male smokers exhibit higher levels of oxidative DNA adducts in their sperm nuclei such as 8-hydroxydeoxyguanosine (8-OHdG), which may be indicative of potential genetic damage (Fraga et al., 1996; Shen et al., 1997). Nicotine (Mol. Wt. 162.0, Fig. a) is one of the major hazardous components of cigarette smoke which mimics most of the deleterious effects of cigarette smoke (Kavitharaj and Vijayammal, 1999). Nicotine is an alkaloid which is strongly alkaline in reaction and also very volatile. It is present together with a number of minor alkaloids, in tobacco and a wide variety of other plants. The structural formula of nicotine shows it to be a combination of pyrrolidine and pyridine rings (Fig. a). The carrier of nicotine namely tobacco leaves may be used for pleasure by smoking it in pipes, cigars or cigarettes or by taking it in unsmoked form as oral and nasal tobacco snuff. Non-smokers are exposed to nicotine through plant material and sidestream tobacco smoke. This means that in human, nicotine is always utilized in the presence of a very large variety of natural compounds or their pyrolysis products, depending on the route of administration. In recent years, the use of nicotine in chewing gum and cutaneous patches has been developed as aid to smoking cessation. The toxic properties of nicotine however, make it useful as an insecticide, which has led to its use in agriculture and horticulture. It has recently been recognized that tobacco consumption may be beneficial in the prevention of Parkinson’s
disease or in alleviating inflammatory bowel syndrome (Gorrod and Wahren, 1993).

There are variety of ways in which nicotine-containing products are administered and the pH dependency of nicotine absorption markedly influence the amount of nicotine which enters the systemic circulation, for instance the smoke from most European cigarettes (flue cured tobacco) is acidic (pH 5.5) and nicotine is in the ionized form and little is absorbed from smoke held in respiratory tract. With the exception of the intravenous route, nicotine administration involves absorption through a cell membrane. In the form of the indissociated base, highly lipid soluble nicotine readily permeates cell membranes. However, differences in pH throughout the body determine the actual amount of nicotine absorbed (Gorrod and Wahren, 1993). Nicotine is rapidly absorbed from inhaled cigarette smoke, reaching the systemic circulation at a rate comparable with intravenous administration. Processes involved in smoking such as depth of inhalation and frequency of puffs are controlled by the smokers to obtain the desired amount of nicotine.

Nicotine directly interacts with membrane components that are normally bound by acetylcholine. As a result of this, the enzyme acetylcholinestrase can not hydrolyse them, thus causing a change in the metabolism of the cell (Guraya, 1987). Furthermore, Calzada et al. (1992) reported the presence of nicotine-type receptors for cholinergic stimulation on the sperm plasma membrane. This is proved by adding nicotine to the human sperm resulting in increased influx of radiolabled lipophilic cation TPMP⁺. Nicotine also induced 20% hyperpolarization to sperm membrane. It is now well established that cytochrome P450s are involved in the oxidative metabolism of nicotine to form its major metabolite viz. cotinine (Gorrod and Wahren, 1993) which is as under:
The nicotine content of tobacco varies from 0.5 to 0.8%. A cigarette usually contains about 2% nicotine, but the absolute amount per cigarette may vary as much as 12 times depending on the brand of tobacco used. About 90% nicotine is absorbed into the lungs from the smoke inhaled, whereas only 10 to 25% is absorbed if the smoke is merely taken in the mouth and expelled (Barar, 1987).

The results of several studies in this area have been contradictory. In one study, enhanced sperm movement has been associated with smoking, at least with the first hour of ejaculation (Saarenen et al., 1987). Nicotine exposure in rats leads to atrophy of the testis and impaired spermatogenesis (Weisberg, 1985). The fact that nicotine and its major metabolite, namely cotinine are detectable in semen (Pacifici et al., 1993; Vine et al., 1993) suggests that perhaps other components of tobacco smoke are also able to enter the semen and affect sperm development.

Blackburn et al. (1996) reported that nicotine has a direct toxic effect on the ovary function to suppress it such as i.e. inhibited ovulation, estradiol production and fertilization. Using a primary culture of testicular cells from hypophysectomized rats it was shown recently that nicotinic-cholinergic agonists inhibited androgen biosynthesis (Kasson and Haueh, 1985). Prenatal administration of nicotine was reported to bring about alterations in the sexual behaviour and testosterone levels of rats (Segarra and Strand, 1989).
Barbieri et al. (1986) have also reported that aromatase in human trophoblast was inhibited in vitro by nicotine, cotinine and anabasine. It has been considered that chronic treatment with nicotine increases the number of its binding sites. Recently, Pauly et al. (1996) carried out exhaustive experiments on the 3Hcr-nicotine binding sites in mouse brain and concluded that the same receptors are affected by agonist or antagonist treatment. To check the gonadotoxic effect, chronic administration of nicotine (subcutaneously, 0.6 mg/kg body weight for 35 days) was found to produce enhanced synthesis of cholesterol, triglycerides, phospholipids and free fatty acids in the liver and testis of rats, but on the other hand the testosterone and estradiol levels in the serum were lower (Kavitharaj and Vijayammal, 1999), this was in agreement with the results in which it was shown that plasma testosterone level is reduced significantly in heavy smokers (Briggs, 1973). Nicotine at a concentration of 1 mM and above, has deleterious effects on sperm motional characteristics, while 0.1 mM concentration failed to have any effect (Reddy et al., 1995). Further, Cope (1998) suggested that the effects of nicotine and/or cigarette smoke in toto on sperm motility are caused by reactive oxygen species (ROS) generated from leukocyte contamination of semen and that these effects can be inhibited by antioxidants.

In another attempt, Crandall et al. (1989) had shown that nicotine at concentrations found in the cervical mucus of female smokers appeared to enhance sperm penetrability in vivo through ovulatory bovine cervical mucus, but sperm motility parameters were not affected by the addition of nicotine to semen samples incubated with BWW medium. The rate of sperm penetration of zona-free hamster egg was shown more significantly to be decreased with the increase in nicotine concentration from 0.1 to 10 mM (Pekarsky et al., 1995). In a series of experiments carried out by Wetscher et al. (1995a,b) it was shown that nicotine induced the positive oxidative stress in esophageal mucosa and pancreatic tissue of rat towards cellular damages.
In addition to above, there are many other agents/drugs which enter the human and animal semen. Though most of these are non-toxic to spermatozoa, while others do have some impacts (Maan and Mann, 1981). Caffeine (Mol. Wt. 194.20. Fig. a) and its two major metabolites namely theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) are three closely related alkaloids in methylxanthine group that occur in plants widely distributed geographically. At least half the human population of the world consume tea (containing caffeine and small amounts of its metabolites), prepared from the leaves of Thea sinensis, a bush native to Southern China, and now extensively cultivated in other countries. Coca and chocolate, from the seeds of Theobroma cacao, contain theobromine and some caffeine, while coffee, the most important source of caffeine in the American diet, is extracted from the fruit of Coffea arabica and related species (Goodman and Gilman, 1996).

Cola-flavored drinks usually contain considerable amount of caffeine, in part because of their content of extracts of the nuts of Cola acuminata (the guru nuts chewed by the natives of the Sudan) and in part because of caffeine as such in their production (Graham, 1978). The basis for the popularity of all the caffeine-containing beverages is the ancient belief that they have stimulant and antisoporific actions that elevate mood, decrease fatigue and increase capacity for work (Jorda et al., 1989; Goodman and Gilman, 1996). In recent years, there has been a resurgence of interest in the natural methylxanthines and their synthetic derivatives, principally as a result of increased knowledge of their cellular basis of action. As cited earlier, caffeine, theophylline and theobromine are methylated xanthines. Xanthine itself is a dioxyxypurine and is structurally related to uric acid. The solubility of methylxanthines is low which is however, much enhanced by the formation of complexes (usually 1:1) with a wide variety of compounds.

Caffeine is an odourless silky substance, sparingly soluble, 1 part in 60 of water but freely soluble in boiling water. The caffeine, and to a lesser extent
Caffeine \( (\text{C}_{8}\text{H}_{10}\text{N}_{4}\text{O}_{2}) \) (1, 3, 7 - trimethylxanthine)

Nicotine \( (\text{C}_{10}\text{H}_{14}\text{N}_{2}) \) [3-(N-Methyl Indole) Pyridine]

Fig. a: Chemical structure of caffeine and nicotine.
theophylline and theobromine, are lipophilic in nature and readily cross biological membranes (Blanchard and Sawers, 1983). Their absorption into the blood stream after oral ingestion is rapid and complete. Caffeine from both tea and coffee is absorbed at approximately the same rate, but because some caffeinated soft drinks have lower pHs and a high sugar content, the rate of absorption is slower (Tarka, 1982; Kincaid, 1982). For caffeine at 20 min after ingestion, the percentage absorption is 9% at pH 2.1 and 22% at pH 7.0 and the clearance is 189 and 301 ml/hr/m², respectively (Chvasta and Cooke, 1971).

The methylxanthines can pass into amniotic fluid and cross the placenta (Tarka, 1982). Caffeine is readily available in coffee (containing 85-110 mg/cup), tea (about 50 mg/cup), cola beverage (30-45 mg/serving), cocoa (5 mg/cup) and chocolate (25 mg/small bar) (Somani and Gupta, 1988).

In North America, the estimated caffeine consumption averages nearly 200 mg per person per day. About 60% of that is consumed in coffee, 16% in tea, 16% in soft drinks, and less than 2% in chocolate (Barone and Roberts, 1984). 1-5% of caffeine is excreted unchanged and the remainder metabolized, primarily in the liver (Bonati and Garattini, 1984). Its mean half-life is about three hours and may range between two and one-half hours to four hours (Statland et al., 1976). It is demethylated about 72% by the hepatic cytochrome P450 1A2 (CYP1A2) enzyme primarily to 1,7-dimethylxanthine (1,7-X: paraxanthine) and to a lesser extent to 3,7-dimethylxanthine (3,7-X: theobromine) and 1,3-dimethylxanthine (1,3-X: theophylline) in human, 20% and 8%, respectively (Lelo et al., 1986). Further demethylation of paraxanthine follows by the same CYP1A2 enzyme results in 1-methylxanthine (1-X) and 1-methyluric acid (1-U) which are the main metabolites of caffeine in blood plasma and urine as well (Kalow and Tang, 1993).

The proposed mechanisms of methylxanthine-induced physiological and pharmacological effects have included (1) inhibition of phosphodiesterase, thereby increasing intracellular cyclic adenosine 3':5' monophosphate (cAMP)
and/or cyclic guanosine 3':5' monophosphate (cGMP), (2) direct and/or indirect effects on intracellular calcium concentrations, (3) antagonism of A1-adenosine receptors (A1 – AdR), (4) Uncoupling of intracellular calcium increases with muscle contractile elements (Goodman & Gilman, 1996). Mammalian sperm motility has been reported as being enhanced and/or prolonged in the presence of caffeine as a phosphodiesterase inhibitor (Garbers et al., 1971; Frenkel, 1973; Schoenfeld et al., 1975; Shilon et al., 1978; Amelar et al., 1980; Makler et al., 1980; Levin et al., 1981; Tash and Means, 1982; Weeda and Cohen, 1982; Aitken et al., 1983a; Babcock et al., 1983; Tesarik et al., 1992; Stachecki et al., 1994; Fayed, 1996; Lopez and Alvarino, 2000).

Stimulation of the kinetic energy and respiration of spermatozoa after in vitro addition of caffeine was first reported to occur in bovine epididymal and ejaculated spermatozoa (Drevius, 1971). Caffeine was later reported to stimulate the motility and forward progression of ejaculated human spermatozoa (Bunge, 1973; Schoenfeld et al., 1973 and 1975; Aitken et al., 1983a; Tesarik et al., 1992).

Caffeine has also been found to increase sperm velocity (Traub et al., 1982; Ruzich et al., 1987; Hammitt et al., 1989; Mbizvo et al., 1993), and increase in the percentage and viability of motile spermatozoa and metabolism when added to human semen (Makler et al., 1980; Levin et al., 1981; Aitken et al., 1983a; Moussa, 1983), the effect being most dramatic with poor samples e.g. cryopreserved ones.

Mammalian spermatozoa must undergo a complex process of maturation called capacitation, before being able to fertilize oocyte. This process takes place under physiological conditions in the female genital tract, although it can be induced in vitro by incubation in various culture media such as defined biological materials e.g. follicular fluid (FF), serum, peritoneal fluid (PF) and defined agents e.g. calcium ionophore A23187, progestrone, adenosine analogues and methylxanthines (caffeine and pentoxifylline)
(Nassar et al., 1998). In many mammals, there are characteristic motility changes associated with sperm capacitation \textit{in vitro} and \textit{in vivo}, which are collectively termed as hyperactivation (HA). The increased motility in hyperactivated spermatozoa aids the sperm cells in accessing to oocyte and penetrating egg investments (Yanagimachi, 1970; 1981; Burkman, 1984; Mortimer et al., 1984; Yanagimachi, 1988; O’Flaherty et al., 1999). The most obvious expression of hyperactivation is a change in the pattern of sperm movement due to the increased bending of the proximal flagellum, the resulting trajectory movement is greatly curved and tortuous (Katz et al., 1986). This suggested that hyperactivated spermatozoa are characterized by a vigorous motility with a high curvilinear velocity (VCL) and low linearity (LIN) (Mbizvo et al., 1993).

The capacitation process is associated with removal of cholesterol which leads to a decrease in the cholesterol: phospholipid molar ratio in the sperm membrane and an increase in the membrane fluidity (Go and Wolf, 1983; Langlais et al., 1988; Hoshi et al., 1990; Benoff, 1993). The amount of phospholipids does not appear to change considerably during the process of capacitation. However, capacitation is associated with an increased phospholipid methylation and increased synthesis of phosphatidylcholine and phosphatidylethanolamine (Llanos and Meizel, 1983).

Capacitation has been shown to involve an increase in the concentration of intracellular calcium \([\text{Ca}^{2+}]\), (Fraser, 1987; DasGupta et al., 1993; Breitbart et al., 1995; Cordoba et al., 1997; Dominguez et al., 1999). In spermatozoa, the intracellular \text{Ca}^{2+} is regulated by the \text{Ca}^{2+}– \text{ATPase} (acting as a \text{Ca}^{2+} extrusion pump), \text{Ca}^{2+}/\text{H}^{+} exchanger system plus \text{Na}^{+}/\text{Ca}^{2+} antiporter (acting as \text{Ca}^{2+} entrance systems) in the plasma membrane and possibly, by the intracellular \text{Ca}^{2+} stores (Serres et al., 1994).

A low molecular weight protein, Caltrin, associated with ejaculated bull and guinea pig spermatozoa (bound in both the head and the tail) inhibits the \text{Na}^{+}/\text{Ca}^{2+} exchanger and maintains the intracellular \text{Ca}^{2+} at low levels and also
prevents from early capacitation in their journey to the site of fertilization (Rufo et al., 1984; Coronel et al., 1990).

Ca\(^{2+}\) channel is characterized as a H-type and L-type voltage-dependent system which has been demonstrated in mammalian spermatozoa for mediating the Ca\(^{2+}\) entry (Beltran et al., 1994; Florman et al., 1995a). Ca\(^{2+}\) influx in capacitated sperm cells can stimulate adenyl cyclase activity, mediated by calmodulin and resulting in an elevation in the production of cAMP (Monks et al., 1986; Toscano and Gross, 1991).

Moreover, the process of capacitation could be induced by addition of biological cAMP analogues (Visconti et al., 1995). CAMP, in turn, activates cAMP-dependent protein kinase (PKA). A possible mediator of the activation of flagellar motility by PKA has been described. A 56,000 Da phosphoprotein of bovine spermatozoa, that is axokinin appears to be the major PKA substrate (Tash et al., 1984). Tash and Means (1982) pointed out that the regulatory role of calcium can be demonstrated in various sperm functions, including a calcium-calmodulin mediated phosphorylation of dynein with the use of a myosin light-chain kinase-type enzyme. This enzyme regulates tubulin-dynein interaction which is the basis of flagellar beating. However, alteration of intracellular Ca\(^{2+}\) as a result of modulation of cellular calcium transport may be a possible mechanism by which methylxanthines like caffeine enhanced the motility in human spermatozoa (Hong et al., 1985).

Calcium influx also triggers the activation of some other enzymes such as calcium-dependent protein kinase (PKC), phospholipases C (PLC) and D (PLD) in mammalian spermatozoa which leads to a series of protein phosphorylation and/or hydrolysis (Furuya et al., 1993). In capacitation, involvement of the generation of free oxygen radicals has also been suggested. Here, these radicals cause tyrosine phosphorylation by protein tyrosine kinase which mediates activation of PLC in spermatozoa (Aitken et al., 1998; de Lamirande et al., 1998).
Exogenous stimulation with caffeine and dbcAMP (dibutyryl analog of cAMP) in monkey resulted in the completion of capacitation through protein tyrosine phosphorylation (Boatman and Bavister, 1984; Vandevoort et al., 1992 & 1994; Mahony et al., 1996; Mahony and Gwathmey, 1999).

Vandevoort et al. (1992 & 1994) have shown that the addition of caffeine and dbcAMP to medium containing monkey spermatozoa resulted in an increase in flagellar bending amplitude and mean amplitude of ALH and also a decrease in linearity (LIN) and straight-line velocity (VSL) as well. Mahony and Gwathmey (1999) reported that phosphotyrosines detected by immunoreactivity method was associated with proteins of principal region of the sperm tail. These phosphotyrosine containing proteins were identified as the major fibrous sheath proteins.

It has been demonstrated that increase of [Ca\(^{2+}\)]\(_i\) in capacitation leads to PLC activation which converts membranous phosphatidyl inositol bisphosphate (PIP\(_2\)) to diacylglycerol (DAG) and inositol triphosphate (IP\(_3\)) (O'Toole et al., 1996). The latter releases Ca\(^{2+}\) from intracellular pools (Breitbart and Spungin, 1997) and the DAG activates phospholipase A\(_2\) (PLA\(_2\)) and PKC which are involved in completion of capacitation and also activate the Ca\(^{2+}\) channels in plasma membrane (Spungin and Breitbart, 1996).

The capacitation process ends with an exocytosis event called the acrosome reaction (AR). In mammalian species, spermatozoa must undergo the AR to penetrate the zona pellucida and fuse with the oolemma (Yanagimachi, 1981; Osman et al., 1989; Ain et al., 1999; Funahashi and Nagai, 2001). The AR is characterized by point fusion between the outer acrosomal and plasma membranes. The term ‘AR’ generally refers not only to the membrane fusion stage, but to subsequent loss of the reacted elements or exocytosis of hydrolytic enzymes outwardly (Yanagimachi, 1988, 1994).

In the absence of any specific stimuli, human spermatozoa can undergo the acrosome reaction process. It has been suggested that self aggregation of sperm receptors for zona pellucida may account for the spontaneous acrosome
reaction (Stock and Fraser, 1987). According to another possibility, the Ca^{2+} pumping outwardly in plasma membrane becomes less efficient with time due to depletion of ATP, for example (Yanagimachi, 1994). Phosphodiesterase inhibitors like caffeine and pentoxifylline (Tesarik et al., 1992; Das Gupta et al., 1994) can enhance the rate of AR in mammalian spermatozoa. It is well known that the relationship between AR and sperm-zona pellucida binding varies from one species to another, for example in guinea pig, only acrosome reacted spermatozoa bind to the oolemma (Yanagimachi, 1981). In mice, only acrosome-intact sperm interact with and bind to oolemma while the AR is induced later, probably by interaction between sperm binding sites and oolemma glycoproteins (Wassarman, 1987). The situation in man is somewhat different because acrosome-intact sperm and acrosome-reacted ones, both have been observed on the zona pellucida (Overstreet, 1976), although it is also known that incubation of human sperm in vivo to achieve capacitation will increase zona pellucida binding (Singer et al., 1985). Inducing the human acrosome reaction with calcium ionophore A23187 decreases sperm-zona pellucida binding with oocytes, therefore induction of AR following binding to the zona pellucida may be more important for the sperm cell which ultimately fertilizes the human oocyte (Liu and Backer, 1990). Furthermore, De Jonge et al. (1991) pointed out that caffeine treatment induced the spontaneous AR in human sperm cells. It is possible that spontaneous loss of acrosome may be more frequent when sperm have abnormal morphology (poor samples) and these sperm may then be unable to bind to the oolemma (Liu and Backer, 1990).

Caffeine in spite of its paramount role in increasing the rate of motility failed to enhance the fertility or prolificacy in rabbits (Lopez and Alvarino, 2000). It was reported that addition of caffeine to the medium containing porcine matured oocytes resulted in an increased rate of sperm penetrations and an abnormally high incidence of polyspermic fertilizations (Niwa, 1993; Funahashi and Day, 1997). Funahashi et al. (2000a) and Funahashi and Nagi
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(2001) demonstrated that caffeine stimulated both capacitation and spontaneous AR in boar spermatozoa followed by a decrease in the monospermic fertilizations.

Caffeine, as a purine alkaloid demonstrates some structural and functional similarities with other endogenous metabolites in body (Fig. b) like: purine, xanthine, uric acid and adenosine.

![Chemical structure of some important purines.](image)

**Fig. b : Chemical structure of some important purines.**

Adenosine affects many of the physiological processes throughout the body via its receptors (AdR) which are generally subclassified into A₁, A₂ and A₃. A₁ and A₃-AdR mediate an inhibition and A₂-AdRs (A₂a and A₂b) cause an activation of adenylate cyclase/cAMP pathway (Nakata, 1990). Fraser and Duncan (1993) reported the presence of stimulatory A₂-AdR on uncapacitated mammalian spermatozoa which could be activated by adenosine and its analogues to stimulate the fertilizing ability by accelerating the capacitation. In human body, caffeine plays an important role as an antagonist to A₁-AdR in a competitive inhibitory manner (Woods, 1991). In 1995, Minelli and his co-workers described the first evidence of A₁-AdR receptors on bovine epidydimal spermatozoa membranes. Minelli et al. (2000) confirmed the presence of A₁-AdR on mammalian sperm cells with an acrosomal localization which functionally coupled to calcium uptake into IP₃-gated stores in such spermatozoa. According to a recent study, it appears that adenosine induces
capacitation via $A_2$-AdR in intact cells and inhibits spontaneous AR via $A_1$-AdR in capacitated sperm cells (Funahashi et al., 2000b).

All aerobic organisms require oxygen for life. Although it is an essential element, the major metabolites of oxygen such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), the hydroxyl radical (OH), and peroxynitrite anion ($ONOO^-$) are capable of adversely modifying cell functions, ultimately endangering the survival of the cell. These reactive oxygen species (ROS) are the major contributory factors in male infertility. The source of ROS is either the defective spermatozoa itself or the infiltrating (contaminating) leukocytes, mostly neutrophils in the seminal plasma (Plante et al., 1994; Sharma and Agarwal, 1996). It may be noted that the production of ROS is a normal physiological process but an imbalance between ROS generation and scavenging activity is detrimental to the cell. Excessive generation of ROS is called as a positive oxidative stress. It is defined as a situation in which there is a shift in the ROS balance towards the pro-oxidants, because of the presence of either excess ROS or diminished antioxidants (Sharma and Agarwal, 1996). ROS therefore, have dual effect on human spermatozoa. Physiologically, ROS can trigger some normal sperm functions in vitro like capacitation and finally acrosome reaction (De Lamirande and Gagnon, 1993; Griveau et al., 1994), but these positive effects are strictly related to the equilibrium between ROS and scavenger systems. However, at a very high concentrations, ROS can affect the various parameters of sperm including sperm motility and morphology. This further leads to poor sperm-zona pellucida binding and sperm-oocyte functions (Aitken and Fisher, 1994; De Lamirande and Gagnon, 1995; De Lamirande et al., 1998).

Somatic cells contain significant amount of antioxidants within their cytoplasm. Spermatozoa however, lose most of their cytoplasm during maturation and thus, lack the endogenous repair mechanisms and enzymatic defenses observed in other cell types. This leaves them at a significant disadvantage. They are protected from oxidative insult by seminal plasma,
which contains an abundance of antioxidant enzymes such as, superoxide dismutase (SOD) GSH peroxidases (GPx) and catalase, which are capable of removing key ROS like $O_2^-$ and $H_2O_2$, and scavengers (non-enzymatic antioxidants) like albumin and taurine (Halliwell & Gutteridge, 1989). Seminal plasma also contain crucial chain-breaking antioxidants such as urates, ascorbate and GSH (thiol groups) (Donnelly et al., 1999). It has been shown that seminal plasma from infertile men has a significantly lower total antioxidant capacity than that from the fertile men (Mazzilli et al., 1994; Lewis et al., 1997). The studies have demonstrated that men with high levels of ROS generation had seven times the less chance of effecting pregnancy in a partner compared to men with low ROS (Sharma and Agarwal, 1996).

Fluidity and flexibility of cell membranes are mainly dependent on their lipid composition. Pioneering studies of the analysis of sperm lipids have shown the effective presence of neutral fatty acids, cholesterol, phospholipids (mainly phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin) and glycolipids in both the mammalian and non-mammalian spermatozoa (Maan and Mann, 1981). Mammalian spermatozoa demonstrate an asymmetric transverse distribution of phospholipids with aminophospholipids preferentially located in the inner and choline-containing phospholipids in the outer leaflet of cell membrane (Israelachvili et al., 1980).

Human spermatozoa are particularly sensitive to free radical assault because of their high content of polyunsaturated fatty acids (PUFA), which can undergo lipid peroxidation (LPO) initiated by ROS. PUFA are required to give the plasma membrane the fluidity it needs to sustain biochemical and biological functions, including the maintenance of ATPase activity and completion of fusion events between sperm and oocyte (Gomez et al., 1998). Almost 50% of the PUFA in human sperm cell is docosahexaenoic acid (DHA, C22:6 n-3) with six unsaturated double bonds per molecule. When the lipid peroxidation cascade is stimulated with a ferrous ion promoter, 60% of this fatty acid is lost from the membrane, mostly from phosphatidyl ethanolamine.
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and ethanolamine plasmalogen (Jones et al., 1979). This loss of docosahexaenoic acid from the membrane is probably the result of fatty acid decomposition rather than enzymatic cleavage by phospholipase A₂ since the latter appears to act largely on the C₂ fatty acid of phosphatidyl choline (Bennet et al., 1987). The best morphological pattern also corresponds to the highest content of DHA in the sperm populations (Gandini et al., 1999). Human sperm cell has an active lipid metabolism which produces a rearrangement of the constitution of essential fatty acids during the spermatogenesis and during sperm maturation processes (Poulos and White, 1973). Aitken et al. (1989) reported that lipid peroxidative damage leads to the loss of membrane integrity, which is strongly associated with the loss of sperm motility. An inverse correlation has been observed between the percentage of motile sperm cells and the quantity of ROS detected in the semen (Iwasaki and Gagnon, 1992). Since lipid peroxidation is expected to deplete functional or normal lipids, there is quite likely to be an increased demand for lipid synthesis under these conditions.

Lipid peroxidation is also known to inactivate many enzymes, both cytosolic and membrane bound in other cells. The list includes enzymes such as glyoxylases, β-amylase, choline oxidase, succinyl oxidase, cytochrome oxidase, trypsin, urease, ribonuclease A, lysozyme, carboxypeptidase A, lactate dehydrogenase, creatine kinase, Ca²⁺-ATPase, cytochrome P450 and glucose-6-phosphatase (Ohata et al., 1989; Thomas and Poznansky, 1990). Lipid peroxidation results in a variety of products capable of damaging the enzymes. A number of aldehydes are formed which can attack the primary amino groups in proteins. Bifunctional aldehydes such as malonyldialdehyde (MDA) can cross link proteins. Hydroxy alkenals, another group of products of lipid peroxidation are known to destroy sulphydryl groups in proteins (Thomas and Poznansky, 1990).

Membrane lipids are known to influence certain protein dependent activities and these proteins have been shown to be intimately associated with
the hydrophobic core of the membrane. Lateral diffusion of lipids in the membrane environment may allow proteins to undergo conformational changes necessary for receptor and transport functions. The fluidity of lipid bilayer is hence, a fundamental tenet of membrane study (Brasitus et al., 1979). The term “lipid fluidity” as applied to model bilayers and natural membranes denotes the relative motional freedom of the lipid molecules or substituents thereof. This term is broad and includes different types of motions, e.g. rotational or lateral diffusion of a molecule in an array, movements of substituent groups of a molecule and flow of the molecules under a pressure gradient in according with fluidity, which is 1/viscosity, of the molecular array (Brasitus and Schyachter, 1980).

Peroxidation of membrane lipids is a physiological phenomenon occurring in all cells that are rich in lipids. The divinyl methane structure present in all polyunsaturated fatty acid is particularly susceptible to hydrogen abstraction, resulting in the formation of a fairly stable free radical

![Divinyl Methane Structure](image)

and, in the presence of oxygen, in the initiation of a radical chain leading to a typical autoxidation reaction (Mead, 1976). It therefore, implies that peroxidation of polyunsaturated membrane lipids leads to a disturbance in the membrane structure and hence its functions. Lipid peroxidation decreases the membrane fluidity or in other words, increases the membrane viscosity. The fluid mosaic structure of the membrane when disturbed, may lead to its dysfunction. Collectively, LPO is a chain reaction whereby molecular oxygen is incorporated into PUFA to yield lipid peroxides. Two general models of LPO
may be defined: enzymatic oxygenation of PUFA, which is catalysed by the 
eicosanoid synthesizing enzymes: cyclooxygenase and lipoxygenase, and non-
enzymatic oxygenation of PUFA. The latter process, named PUFA 
autoxidation, is stimulated by ROS and transition metals and limited by 
antioxidants such as vitamins (Janero, 1990).

Lipid peroxidation involves the initiation and propagation stage and 
occurs by the Haber-Weiss reaction and/or the Fenton reaction (Fig. c). Both 
reactions generate the hydroxyl radical, which then initiates lipoperoxidation. 
By itself, neither O₂ nor H₂O₂ is energetic enough to initiate LPO directly but, 
in the presence of catalytic amounts of transition metals, such as iron or 
copper, they react and form the OH radical (the Haber-Weiss reaction). In the 
Fenton reaction, the hydroxyl radical can be directly generated from H₂O₂ if 
ferrous ions and an alternating reducing agent such as ascorbate are present. 
(Halliwell & Gutteridge, 1989) (Eqn. 1-3):

Fe³⁺ + O₂ → Fe²⁺ + O₂⁻ (O₂⁻ reducing the iron salt). \hfill (1)

Fe²⁺ + H₂O₂ → Fe³⁺ + OH + OH⁻ (Fenton reaction). \hfill (2)

Net: O₂⁻ + H₂O₂ → O₂⁻ + OH + OH⁻ (Haber-weiss reaction) \hfill (3)

To complete the Fenton reaction, a constant source of H₂O₂ is required, 
and the human ejaculate contains all the components (defective spermatozoa 
and contaminating neutrophils) needed to initiate and propagate this free 
radical cascade and lipoperoxidation (Iwasaki and Gagnon, 1992; Kessopoulou 
et al., 1992).

Following the initiation stage of lipid peroxidation cascade, the extent 
to which the process proceeds will depend on the antioxidant strategies 
employed by the spermatozoa. The hydroxyl free radical (OH) has sufficient 
reactivity to abstract a hydrogen atom from a lipid molecule (Eqn.4). Since 
hydrogen atom has only one electron, this leaves behind an unpaired electron
on the carbon atom. The carbon atom in a polyunsaturated fatty acid tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which rapidly reacts with $O_2$ to give a hydroperoxy (peroxyl) radical (Eqn.5). This radical then abstracts hydrogen atoms from other lipid molecules and so continue the chain reaction of lipid peroxidation (Eqn.6). The hydroperoxy radical combines with hydrogen atom that it abstracts to give a lipid hydroperoxide (Eqn.6).

\[ \text{Lipid}_H + \text{OH} \rightarrow \text{Lipid} + \text{H}_2\text{O} \quad (4) \]
\[ \text{Lipid} + \text{O}_2 \rightarrow \text{Lipid}_2\text{O} \quad (5) \]
\[ \text{Lipid}_2\text{O}_2 + \text{Lipid}_H \rightarrow \text{Lipid}_2\text{O}_2\text{H} + \text{Lipid}^\cdot \quad (6) \]

Lipid peroxides are extremely cytotoxic to human spermatozoa as a result of the decomposition of these peroxides into highly toxic aldehydes that migrate from one site to another in the body (owing to their long life), thus propagating the injuries. Inhibition of membrane-bound enzymes, especially ATPase is known to result due to the accumulation of lipid peroxides. Lipid peroxidation also impairs cell membrane ion exchange that is essential for maintaining normal sperm motility (Rao et al., 1989). Normally, the lipid hydroperoxides tend to accumulate and stabilize in plasma membrane, unless a transition metal is added to the cell suspension, which will result in a sudden acceleration of LPO and loss of most of cell functions, called propagation phase of LPO (Fig. c). Resulting substances in this phase are peroxyl ($\text{Lipid}_2\text{O}^\cdot$) and alkoxyl ($\text{Lipid}_-\text{O}^\cdot$) radicals (Eqn. 7-10) that attack adjacent PUFAs and propagate the LPO cascade. As a consequence of these chain reactions, peroxidation damage spreads through the plasma membrane unless terminated by chain-breaking antioxidant such as vitamin E, (Griveau & Le Lannou 1997) (Fig. c):

\[ \text{Lipid}_2\text{O}_2\text{H} + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{Lipid}_-\text{O} \quad (7) \]
Lipid_\text{O}_2\text{H} + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{H}^+ + \text{Lipid}_2 \text{(8)}

2 \text{Lipid}_2 \rightarrow \text{O}_2 + [\text{Lipid}] \text{O} = \text{O} [\text{Lipid}] \text{(9)}

\text{Lipid}_2 + \text{Lipid} \rightarrow [\text{Lipid}] \text{O} = \text{O} [\text{Lipid}] \text{(10)}

So, oxygen uptake is given by Eqn.11 (Kovalski \textit{et al.}, 1992),

\begin{equation}
\frac{-d\text{O}_2}{dt} = K_p [\text{Lipid} - \text{O}_2] [\text{Lipid} - \text{H}] \text{(11)}
\end{equation}

Malonyldialdehyde (MDA) is an end product of LPO in the spermatozoa and is known to be induced by ferrous-ascorbate promoter system. This can be measured by the TBA assay and can serve as an important diagnostic tool in LPO measurement (Aitken \textit{et al.}, 1989). The MDA concentration exhibits excellent inverse relationship with the sperm-oocyte fusion (Aitken \textit{et al.}, 1992).

In biological systems there are two types of defense mechanism against the production of ROS. One type of defense mechanism that accounts for primary line of defense is the catalytic removal of ROS by antioxidant enzymes such as superoxide dismutase (SOD), catalase and peroxidase. SOD lowers the steady-state of O$_2^-$ while catalases and peroxidases do the same for H$_2$O$_2$. Iron containing (Fe-SOD) and manganese-containing (Mn-SOD) enzymes are characteristic of prokaryotes. In eukaryotic cells, the predominant forms are the Cu-SOD and Zn-SOD that are located in the cytoplasm. Mn-SOD is found in mitochondrial matrix (Fridovich, 1986). Since SOD is the scavenger of O$_2^-$, therefore the biosynthesis of SOD is controlled by O$_2^-$ (Fridovich, 1986).

It is well documented that higher the SOD activity, higher the progressive motility. So, SOD activity could be used as an indicator of the amount of damage to the plasma membrane as well as to the cellular viability (Beconi \textit{et al.}, 1991). Extracellular SOD binds to the neck region of a sub-group of spermatozoa in semen and these cells can remain motile for a period longer than spermatozoa without bound SOD (Storey, 1997) (Fig. c). It has
been demonstrated that seminal catalase (EC. 1.11.1.6) is originated from prostate, however, its activity is not correlated with the usual prostatic markers such as zinc (Jeulin et al., 1989). H₂O₂ as the most toxic ROS owing to its capacity to cross membranes, inhibits enzymes such as G6PDH, SOD, glutathione peroxidase (GPx) and decreases PUFA concentration (Griveau et al., 1995). Since the level of catalase in human spermatozoa and seminal plasma is low the GPx is the main factor that can remove the H₂O₂ generated (Inoue et al., 1989).

\[
2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \text{ (or possibly singlet } O_2) \\
2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2
\]

GPx (EC. 1.11.1.9) is a selenium containing enzyme referred to as GPx1 or classical Se-GPx (tetrameric) and also as GPx4 or phospholipid hydroperoxide GPx (PHGPx, monomeric) with reduced glutathione (GSH) as the electron donor, and can remove H₂O₂ from media as well as peroxyl (ROO') radicals from various peroxides (Calvin et al., 1981; Godeas et al., 1997) (Fig. c).

GSH also serves as a substrate for glutathione S-transferases (GST) (Habig et al., 1974). Glutathione S-transferases (EC. 2.5.1.18), a multigene family of structurally related proteins, are important enzymes of glutathione metabolism. These enzymes catalyse the conjugation of xenobiotics and endobiotics with GSH. In addition, GSTs also demonstrate a selenium independent peroxidase activity (Jakoby, 1978). These enzymes constitute a superfamly of multifunctional proteins (Lai and Tu, 1986). Classification of GSTs into five classes viz. alpha (basic), mu (near neutral), Pi (acidic), theta and microsomal GST have been proposed by Mannervik et al. (1992). Each isozyme of GST is further divided into different subunits. These subunits can be homodimers or heterodimers. Based on the deduced amino acid sequences of cytosolic GSTs, about 30 percent homology is observed between subunits belonging to different classes (Mannervik et al., 1992).
Exogenous Production

\[ \cdot O_2^- \rightarrow H_2O_2 \]

\[ Fe^{2+} \]

Fenton reaction

\[ \cdot OH \]

Scavenging by antioxidants

\[ \cdot OH \]

Fe^{3+}

LPO initiation

ROH + H_2O

2H_2O

Lipid- \( \cdot O_2^- \)

Alkoxy

Lipid-\( \cdot O_2^- \)

Peroxy

Neutralization & stabilization

Antioxidants

LPO termination

\[ \text{Catalase} \]

\[ 2H_2O + O_2 \]

\[ \text{SOD} \]

Endogenous Source

\[ H_2O_2 \]

\[ \text{Fe}^{2+} \]

\[ \text{GRD} \]

\[ \text{G6-PDH} \]

\[ \text{GPX} \]

\[ \text{GSSG} \]

\[ \text{NADPH} \]

\[ \text{NADP}^+ \]

\[ \text{G6-Pho.} \]

\[ \text{G6-PGDN} \]

\[ \text{pentose phosphate cycle} \]

Fig. C: Schematic representation of the major pathways of lipid peroxidation (LPO) triggered by ROS, \( H_2O_2 \), induced LPO, and some enzyme activities. \( H_2O_2 \) would permit, in synergy with the endogenous production of ROS, the accumulation of toxic lipid hydroperoxide and the development of LPO. The recycling of GSH here is done by GRD and needs NADPH as a reducing co-factor from action of G6PDH in pentose phosphate cycle. SOD: superoxide dismutase; GPX: glutathione peroxidase; GRD: glutathione reductase; G6PDH: glucose-6-phosphate dehydrogenase; G6-Pho: glucose-6-phosphate; 6-PGN: 6-phosphogluconolactone; GSH: glutathione (reduced); GSSG: glutathione (oxidized).
The secondary or last line of defence to cell against oxidants/ROS is non-enzymatic antioxidant activity that directly quenches radicals from medium (radical-trapping agents) such as vitamins E and C, small molecular weight compounds like glutathione and albumin. These agents scavenge radicals to terminate free radical reactions and prevent chain propagation reactions. The chain-breaking agents also fall under two types regarding to their function in the medium, one is water-soluble chain-breaking antioxidants such as ascorbic acid (vitamin C), glutathione, albumin and water soluble analogue of vitamin E, Trolox, which scavenge ROS in aqueous phase, whereas another type which are lipophilic chain-breaking antioxidants such as vitamin E (Alpha-tocopherol) which scavenge ROS within the membrane (Russo et al., 1998).

The discovery that lipid peroxidation is a causative mechanism in the aetiology of defective sperm function is important because it leads logically to the use of antioxidant as a strategy to reverse the damage caused by oxidative stress (Aitken and Clarkson, 1988). An effective antioxidant can successfully be used to reverse the oxidative damage inflicted on human spermatozoa during sperm preparation procedure that involve centrifugation of ejaculated cells in the absence of protective environment normally provided by seminal plasma (Aitken, 1994, 1995).

Ascorbic acid (AA) is reversibly oxidized in the body to dehydroascorbic acid. The latter compound possess full Vit. C activity. Orange and lemon juices are outstanding sources of AA and contain approximately 0.5 mg/ml of the vitamin. AA is readily destroyed by heat, oxidation and alkali. AA is present in blood plasma and is ubiquitously distributed in the cells of the body. The white blood cells of healthy adults have concentrations of about 27 µg of AA per 10⁹ cells (Vallanace, 1979). Concentrations of AA in plasma vary from 0.15 to 0.50 mg/dl. The body store of AA is approximately 1500 mg. The renal
threshold for AA is about 1.5 mg/dl of plasma (Goodman and Gilman, 1996). To maintain a body store of 1500 mg of AA or more in an adult man, it would thus be necessary to absorb approximately 60 mg daily (Baker et al., 1971). In seminal plasma, ascorbic acid concentrations are 10-fold higher than in serum (Jacob et al., 1992). Presence of ROS in the semen samples resulted in a significant decrease in the AA (ascorbate) concentrations (Lewis et al., 1997). With a pharmacological supplementation of Vit. C (1g/day), a more than 2-fold increase in plasma AA concentrations can be achieved (Wen et al., 1997). Furthermore, it has been suggested that ascorbic acid is a protective vitamin in the epididymis which prevents spermatozoa against endogenous oxidative DNA damage (Fraga et al., 1991). The amount of DNA damage is significantly greater in infertile male patients than in the control subjects (Kodama et al., 1997). It may be postulated that the patients with DNA damage may benefit from antioxidant treatment. Ascorbic acid in concentrations below 1000 μM protected the human spermatozoa from free radical/ROS damage as evidenced from improvement in their motility and viability (Verma and Kanwar, 1998). Ascorbate and Vit.E (α-tocopherol) work synergistically to protect against LPO, with ascorbate recycling α-tocopherol, allowing it to function again as a free radical chain-breaker (Doba et al., 1985; Buettner, 1993).

The ubiquitous tripeptide, reduced glutathione (L-gamma-glutamyl-L-cysteinylglycine; GSH) functions as a vibrant antioxidant in cells by donating one hydrogen atom from two molecules to a toxic substance spontaneously and/or enzymatically (Meister and Anderson, 1983). Glutathione is widely distributed in animal and plant cells and in micro-organisms. It is found predominantly intracellularly and in relatively high concentrations (0.5 - 10mM). Glutathione has two characteristic structural features: a sulphydryl (-SH) group and a gamma-glutamyl linkage. It is usually the most abundant intracellular thiol (-SH) as well as the most abundant gamma-glutamyl compound (except perhaps for glutamine, which also occurs extracellularly) suggesting that this tripeptide has paramount biological functions (Meister and Tate, 1976).
The intracellular compartmentations of GSH including the nucleus, mitochondrial matrix and endoplasmic reticulum have many paramount implications for cells that are exposed to toxic compounds or to other stresses (Smith et al., 1996). It is noteworthy that there is a kinetically distinct pool of GSH in the nucleus, estimated to be 5 to 10% of total GSH which may be concentrated at the nuclear membrane surface (Loh et al., 1990; Britten et al., 1991). When an adequate stimulation is made, it might be released and added to the cytoplasmic pool to act further. Glutathione is present in the oxidized form (GSSG; disulfide), which is readily converted to the GSH form by glutathione reductase (GRD) using NADPH produced by the glucose-6-phosphate dehydrogenase. It has been reported that glutathione is present mainly in its reduced form in biological tissues at concentrations as high as 2180 μg/g of tissue and in form of GSSG to be present in much smaller concentrations, ranging from 0 to 288 μg/g of tissue (Hissin and Hilf, 1976). Human spermatozoa contain approximately 7 nmoles GSH per 10^8 sperm cells (Storey, 1997).

Glutathione peroxidase catalyses the interaction of glutathione with hydrogen peroxide and other peroxides to yield glutathione disulfide. A number of investigations support the view that glutathione protects cell membranes and proteins by maintaining essential-SH groups and interacting with peroxides and free radicals (Meister and Tate, 1976). Oxidation of ascorbic acid to dehydroascorbic acid produces both ascorbyl radicals and H_2O_2 which can be removed by GSH and GPx in cells (Inoue et al., 1989). Its thiolic group can react directly with H_2O_2, O_2^- and OH and also neutralizes alkoxyl radicals and hydroperoxides producing alcohols, enzymatically (Mann, 1964).

It is generally thought that glutathione (GSH) plays a fundamental biological role. This belief is based on a large variety and number of investigations. The functions that have been ascribed to glutathione include (a) maintenance of the –SH groups of proteins and other molecules; (b) destruction of hydrogen peroxide, other peroxides and free radicals; (c) catalyst for disulfide exchange reactions; (d) coenzyme for certain enzymes (e.g. glyoxalase); (e) detoxification of foreign compounds (e.g. by the
mercapturic acid pathway); (f) translocation of amino acids (and possibly also peptides and amines) across cell membranes (Meister and Tate, 1976).

The major organs involved in the inter-organ circulation of GSH are the liver and kidney, but undoubtedly other organs also participate. Studies on anephric animals treated with transpeptidase inhibitors show that about 67% of the plasma GSH is used by the kidney and the remainder by extrarenal transpeptidase (Griffith and Meister, 1979). These in vivo investigations indicate that GSH is normally translocated to membrane-bound transpeptidase as a discrete step in the gamma glutamyl cycle which is also supported in vitro (Meister and Anderson, 1983).

Glutathione has a number of physiological and pharmacological qualities that act against lipid peroxidation of the cell membrane. Glutathione therapy has been proposed in various pathological situations in which reactive oxygen species could be involved in idiopathic infertility. Marked improvement in total sperm motility and morphology occurring during glutathione therapy suggests that glutathione could act indirectly on spermatozoa by improving the metabolic conditions of the epididymal and testicular structures (Frei et al., 1990). Glutathione acts as a free radical scavenger and results in improved semen quality. Baker et al. (1996) have also demonstrated the effectiveness of glutathione alone or in combination with hypotaurine which is able to react directly with cytotoxic aldehydes produced during lipid peroxidation and thus protects the thiol groups on the sperm plasma membrane (Seligman et al., 1994). It may also facilitate the antioxidant action of alpha-tocopherol in the seminal plasma by participating in the regeneration of this vitamin from tocopheryl radicals. The administration of glutathione has been shown to improve the sperm motility and morphology in infertile men with abnormal semen quality associated with varicoceles or gential tract inflammation (Lenzi et al., 1994).

The antioxidant system is known to work 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. If the function of these enzymes is insufficient for complete elimination of hydrogen peroxide, the Fenton reaction takes place in the presence of transition metals with the subsequent production of toxic hydroxyl radicals. Vitamin E is the chain-breaking antioxidant in membranes, and its oxidation produces the tocopheryl radical, which can then be reduced by ascorbic acid (Vit.C). The oxidation of vitamin C gives rise to ascorbyl radicals which in turn can be reduced by GSH, producing thioly radicals and oxidized glutathione can be regenerated by GSH reductase and peroxidase. 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 (Poli et al., 1993).

α-Tocopherol is classified as a chain-breaking antioxidant because of its ability to break the lipoperoxidative chain reaction through its reaction with lipid peroxyl and alkoxyl radicals in the media (Aitken, 1995). It is a powerful antioxidant (Barclay et al., 1984), and has been shown to afford mammalian cell some protection from oxidative attack both in vivo (Mickle et al., 1989) and in vitro (Wu et al., 1990). α-Tocopherol is present in small but consistent quantities in seminal plasma ranging from 0.08 to 0.90 μ mol/l (Lewis et al., 1997). However, these trace amounts may be adequate, because ascorbate with abundant amount in seminal plasma can regenerate α-tocopherol (Kogan et al., 1992). Oral administration of vitamin E has been shown to improve significantly the in vitro function of human sperm cells as assessed using the zona-binding test (Kessopoulou et al., 1995).

Trolox (6-hydroxy tetramethylchroman-2-carboxylic acid) as a chromanol type antioxidant is an aqueous analogue of Vit.E, lacking phytol chain. Basically, each molecule of trolox can trap two peroxyl radicals at or near the polar aqueous region, when oxidation is initiated in lipid phase (Barclay and Vinqvist, 1994).

Following supplementation, trolox penetrates the lipid bilayer sufficiently to trap the polar peroxyl radicals. An antioxidant that is more...
hydrophilic and resides wholly in the aqueous phase might not be as effective as trolox. Trolox could inhibit Azabis, amidinopropane (ABAP) – induced dilinoleoylphosphatidyl choline peroxidation at pH 4 or 7. The different antioxidant activity of trolox was rationalized in terms of a peroxyl-radical diffusion model as well as specific charge interactions between antioxidants and membrane surface (Barclay and Vinqvist, 1994). Earlier reports also demonstrated that trolox very efficiently traps membrane peroxyl radicals, which may be a result of the greater hydrophobic character of this molecule (Doba et al., 1985). Brzezinska-Slebodzinsk et al. (1995) reported that 10 μM concentration of trolox is sufficient to depress the iron-ascorbate dependent peroxidation of lipids to the control level in boar seminal plasma. In 2000, Lee demonstrated that trolox is able to protect LDL system from oxidation significantly by reducing the thiobarbituric acid reactive substances (TBARS) and other conjugated dienes like peroxyl radicals by about 43 and 80%, respectively. The relative peroxyl quenching capacity of trolox, uric acid and ascorbic acid was calculated to be 1 : 0.86 : 0.47 on a molar basis (Lee, 2000). Trolox was successfully tested in the maintenance of plasma membrane components against oxidation processes after cryopreservation (Wu et al., 1990). Cao and Cutler (1993) suggested that net hydroxyl radical – absorbing capacity of trolox increased with increasing concentrations of the vitamin, but only when the concentration was in the range of 0.1 – 20 μM. The regeneration of phenoxyl radical (derived from chain-breaking antioxidants) by the water-soluble vitamin C can occur with the α-tocopherol radical, which must reside largely in the lipid layer, as well as with the radical from trolox, which must reside principally in the aqueous phase (Doba et al., 1985). The study carried out by Comporti (1989) has shown that trolox also protects the cells against lipid peroxidation in vivo.