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Lipid Peroxidation

Oxygen ($O_2$) is a double-edged sword. Nobody can live without that, but at the same time, there is a darker side to the story as we are continuously exposed to oxygen toxicity. During evolution, the switch from energy generation by anaerobic metabolism to the use of $O_2$ as the electron sink was one of the most important events. The danger occurs only when a single electron is acquired by the $O_2$ molecule to produce the superoxide anion radical ($O_2^-$), an extremely reactive corrosive chemical agent. The unpaired electron acquires a partner by attacking a covalent bond of another molecule. The toxicity of $O_2$ is varied by type of organism used, age, physiological circumstances and diet such as the presence of antioxidant vitamins (Halliwell and Gutteridge, 1984).

In 1981, Gilbert in his book clearly suggested a hypothesis in which it was proposed that many of the damaging effects of $O_2$ could be attributed to the formation of reactive oxygen species (ROS) that possess a key role in the generation of some disease and disorders. Thereafter, other investigators set the seal on the importance of oxygen toxicity and its negative impact on most cells, particularly spermatozoa and their role in certain aspects of male infertility (Halliwell and Gutteridge, 1989; Aitken, 1995; Gomez et al., 1998).

Irrespective of the clinical diagnosis and semen characteristics, the presence of seminal oxidative stress in infertile men suggests that ROS may play a major role in the pathophysiology of male infertility (Pasqualotto et al., 2000). ROS production is also an energy-dependent process (Griveau et al., 1998). A continuous decline in sperm viability in association with an increased ROS production is reported (Kobayashi et al., 2001).

One of the major manifestations of ROS-induced damage in cell populations is lipid peroxidation (LPO). Spermatozoa, unlike other cells, are unique in structure, function and susceptibility to damage by lipid
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peroxidation (Alvarez et al., 1987). In general, the most significant effect of LPO in all cells is the perturbation of membrane (cellular and organellar) structure and function (transport processes, maintenance of ion and metabolite gradients, receptor-mediated signal transduction) (Guraya, 1987 and Emster, 1993). Besides membrane effects, LPO can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl and alkoxyl radicals) or through covalent binding to malonyldialdehyde (MDA) resulting in strand breaks and cross-linking as well (Emster, 1993). ROS can also induce oxidation of critical -SH (sulfhydryl) groups in proteins and DNA which, in turn, will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (Aitken et al., 1994). The oxidative damage to mitochondrial DNA and membranes is known to occur in all aerobic cells which are rich in mitochondria (Kamat and Devasagayam, 2000).

In view of the pivotal role played by lipid peroxidation in mediating free radical damages to cells, in the present study antioxidant potential of ascorbate (AA), glutathione (GSH) and trolox alone/ in combination against nicotine and caffeine-induced peroxidation in human sperm cells have been investigated. These experiments were based on the measurement of thiobarbituric acid reactive substances (TBARS) content expressed as malonyldialdehyde (MDA) which makes a yellow coloured complex with TBA at 90°C. MDA is a stable peroxidation product of polyunsaturated fatty acids (PUFA), usually shows covalent binding and cross-linking to proteins and DNA as well (Emster, 1993). MDA may be viewed as a measure of the cumulative lipid peroxidation that has occurred within the short lifetime of the sperm cells (Warren et al., 1987; Emster, 1993). MDA has also been shown to destabilize the lipid asymmetry in biomembranes (Yuli et al., 1981). Lipid peroxidation seems to be a late stage in the ROS-induced oxidation process. The onset of LPO in susceptible spermatozoa leads to progressive accumulation of lipid hydroperoxides in the cellular and organellar
membranes and, finally, damage to sperm cells (Engel et al., 1998). MDA formation varies considerably from one sample to another (Rao et al., 1989; Engel et al., 1998). Malonyldialdehyde as an endpoint and a small molecular mass degradation product of the peroxidative process can be readily measured by virtue of its capacity to form adducts with TBA and considered as an index of LPO damage to sperm cells (Aitken et al., 1993). Unfortunately, MDA only accounts for around 50% of the products generated during lipid peroxidation, probably derived from the breakdown of cyclic endoperoxides (Marshall et al., 1985; Gomez et al., 1998).

The extent of TBARS/MDA formation in the human ejaculated spermatozoa suspended in PBS (pH 7.2) was monitored for at least 30 min at 37°C to measure the spontaneous, drug (nicotine & caffeine) – treated, and iron-catalysed LPO rates with respect to antioxidant modulations. In our study, various kinetic parameters have also been calculated and summarized using different graphical presentations.

In a set of experiments, various concentrations of drugs as well as antioxidants were used to evaluate the rate of LPO process in the human spermatozoal suspensions. Caffeine (1-15 mM) increased the rate of MDA formation in the sample at the concentrations beyond 5 mM. A strong and positive correlation between caffeine concentration and MDA levels was established (r=+0.831). Caffeine could possibly induce the peroxidation in the spermatozoal membrane fatty acid chains and thereby elevated the TBARS production in the assay mixture. At a very high concentration of caffeine (9mM), the rate of MDA production was also the highest (47.21%, p<0.01).

It has also been found that, the extent of spontaneous LPO in the human ejaculated spermatozoal suspensions upon addition of a concentration range of nicotine (from 0.25 to 2.5 mM) was raised markedly, with a strong r value of +0.920. The highest concentration of nicotine (2.5 mM) imposed a severe peroxidation to sperm cell membranes by about 74.43% (p<0.001) as
compared to untreated samples. It appears that the nicotine could act as a potent oxidant in a dose-dependent fashion in the spermatozoal membranes.

A number of independent lines of evidence suggest a role of lipid peroxidation in the etiology of defective sperm functions (Jones et al., 1979; Aitken and Clarkson, 1988; Aitken et al., 1989; Kovalaski et al., 1992; Oehninger et al., 1995; Donnelly et al., 1999; Potts et al., 2000; Rhemrev et al., 2001). Human spermatozoa are particularly susceptible to peroxidative damage due to containing an extremely high concentrations of PUFA (predominantly 22:6), exhibit no capacity for membrane repair, and possess a significant ability to generate ROS, chiefly superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (Jones et al., 1979; Alvarez and Storey, 1989; Aitken et al., 1993; Grivica et al., 1995 and Gomez et al., 1998). Since caffeine and nicotine promoted the LPO these agents may be involved in generating the ROS when added to sperm samples. The seminal plasma and normal motile spermatozoa do not produce high levels of ROS under the normal conditions. In seminal medium, there are three major sources of ROS: immotile or morphologically abnormal sperm cells, infiltrating leukocytes, and morphologically normal but functionally abnormal spermatozoa (Aitken and West, 1990; Iwasaki and Gagnon, 1992; Plante et al., 1994 and Sharma and Agarwal, 1996). ROS can be generated during normal metabolism (and more in case of disorders) by the electron transport chain, a leak of small proportion of electrons to oxygen, one at a time, resulting in O$_2^-$ formation. It is calculated that liver mitochondrion normally produces about 3x10$^7$ superoxide anions per day (Ritcher et al., 1995). Aitken and Clarkson (1987) proposed that the production of ROS by spermatozoa is partially dependent upon normal or excessive function of a membrane-bound NADPH-oxidase. A global lipid peroxidation may indicate a switch between reversible alterations due to the early inhibition of energy metabolism and irreversible structural degradations (Griveau et al., 1995). A positive correlation between MDA levels and the percentage of sperm cells with morphological stress pattern has been
established (Laudat et al., 1999). The generation of reactive oxygen species is also considered as a common feature of raphidophycean flagellates, causing toxic red tide phytoplankton, (Oda et al., 1997). Ichikawa and his co-workers (1999) demonstrated that lower ROS levels in the human semen may have a role in acrosome reaction (AR) process but excessive ROS may exert a negative impact on plasma membrane as well as AR. Membrane integrity and its proper functioning are basic characteristics of the sperm membranes including cellular recognition and information transduction during cell-cell communications (Nivsarkar et al., 1998).

Changes in the fatty acid composition of membranes as well as the amount of individual sterols account for the change in fluidity. The membrane-associated factors governing lipid mobility in biological membranes include cholesterol content, phospholipid composition, and protein content of the membrane bilayer (Golan et al., 1984). Peroxidation of membrane lipids specially the PUFA, leads to disturbance in the membrane fluidity and hence its function, in other words lipid peroxidation decreases the fluidity and increases the viscosity of membrane (Jain et al., 1993). In order to examine the effectiveness of antioxidants in quenching the free radicals in assay mixture, certain antioxidants namely ascorbate (0.6 – 2.0 mM), trolox (10-30 μM), and glutathione, GSH, (2-10 mM) in controlling the rate of spontaneous LPO in spermatozoal samples, were supplemented. It appeared during the present experimentation that ascorbate at 0.6 to 1.44 mM concentrations acts as an effective antioxidant for the sperm cells suspended in PBS (pH 7.2). At the higher concentrations ascorbate supplementation did not remain effective. The r value was +0.232. It was well reported earlier that ascorbic acid is the only chain-breaking antioxidant found in reduced concentrations in seminal plasma of infertile men like asthenozoospermic individuals (Lewis et al., 1997). This depletion has been suggested to be due to its utilization as a free radical scavenger. Since ascorbate is present at high concentration in the normal seminal plasma, it may protect sperm from oxidative assault in the same way. 

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as it safeguards somatic cells in the blood plasma (Frei et al., 1990 and Hsu et al., 1998). Also, a depleted ascorbate levels is observed in the presence of high levels of ROS and oxidative stress (Lewis et al., 1997). It may, therefore, be safely labeled as an effective antioxidant against oxidative assault.

The ubiquitous tripeptide glutathione (GSH) is widely distributed in animal, plant cells, and in microorganisms. The multifunctional properties of glutathione have been reported in biological systems (Meister and Anderson, 1983 and Donnelly et al., 2000). Glutathione therapy has been proposed in various pathologic situations in which ROS could be involved in idiopathic infertility (Sharma and Agarwal, 1996). The sulphydryl (-SH) constituents of semen (cysteine, GSH and ergothioneine) have been shown to play a paramount role in maintaining sperm functions and metabolism in experimental conditions (Mann and Mann, 1981). In isolated spermatids, GSH can prevent the damage due to exposure to peroxidazing agents (Den Boer et al., 1990). In different biological systems, the GSH redox cycle plays an important role in protecting the cells against oxidative damage. Generally, GSH is present in millimolar concentrations in cytosol and the nucleus of sperm cells, whereas its concentration is low in blood serum and other biological fluids (Daunter et al., 1981; Meister, 1983). Also, glutathione acts to preserve – SH groups of proteins in the reduced state by means of disulfide interchange, although the contribution of GSH is small relative to the total thiol content of the sperm cells (Mann and Mann, 1981). The tripeptide was used to check its effectiveness in scavenging the oxygen free radicals and lipid hydroperoxides of human spermatozoal samples with spontaneous LPO formation. It was registered that there was a negative correlation (r = -0.687) between increasing dose of GSH and MDA levels in samples. The effective dose of GSH was calculated at 5.30 mM which was the intersecting point of a biphasic fashion of action in spermatozoal samples. It has been suggested by Baker et al., (1996) that thiol-containing molecules, such as reduced glutathione and N-acetylcysteine (NAC), can exhibit many different kinds of
antioxidant activity, serving sacrificial antioxidants when used in high concentrations or acting as a substrate for the peroxide decomposing enzyme, glutathione peroxidase (GPx). It has been shown that GSH could improve and partially reverse the functional parameters and also cell membrane characteristics which underwent lipoperoxidation in the dyspermic individuals (Lenzi et al., 1994).

The present work was also designed to work out the antioxidant potential of trolox, a water-soluble analogue of vitamin E, in human ejaculated spermatozoa, against spontaneous and drug-induced LPO in cooperation with ascorbate and glutathione. It was found that trolox with a correlation coefficient of -0.184 diminishes the levels of MDA in spermatozoal samples resulting in inhibition of more breakdown of pre-existing membrane lipid hydroperoxides. Trolox was found to exert a biphasic trend in the reaction of combating against oxygen free radicals in the medium and the intersecting point was found to be 21.4 μM. α-tocopherol (Vit. E) is known for its “chain-breaking” capacity because it breaks the lipoperoxidative chain reaction by interacting with lipid peroxyl and alkoxy radicals (Aitken and Fisher, 1994). α-tocopherol is also reported to act as an antioxidant by breaking the free radical chain reaction and by forming a relatively stable tocopheroxyl radical (Jones and Mann, 1976; Niki et al., 1989). Vitamin E is the primary lipid-soluble, small molecule antioxidant in biological systems and present in small amounts (0.08-0.90 μmol/L) in seminal plasma (Lewis et al., 1997). The present results clearly indicate the role of trolox, an aqueous analog of α-tocopherol, as a potent antioxidant. Damage caused by iron-catalyzed peroxidation is known to be prevented by including α-tocopherol in the medium (Aitken and Clarkson, 1988). In the rat spermatozoa, supplementation of α-tocopherol to lead-induced ROS formation caused a reduction in the levels of free radicals and increased the sperm-oocyte penetration rate significantly (Hsu et al., 1998). A wide range of α-tocopherol concentration has been detected in human sperm cells from 10 to 245 ng/10⁸
spermatozoa and can play a role in association with the antioxidant enzymes, for preserving the functional competence of spermatozoa which have been subjected to an oxidative attack (Therond et al., 1996). The accumulation of \( \alpha \)-tocopherol radicals in medium might lead to a shift from antioxidant to pro-oxidant ability for \( \alpha \)-tocopherol to display a novel action termed tocopherol-mediated peroxidation (Upston et al., 1999). GSH and ascorbate can maintain the levels of reduced \( \alpha \)-tocopherol in medium by reacting with tocopheroxyl radicals to potentiate the inhibition of lipoperoxidation process (Leedle and Aust, 1990; Sharma and Agarwal, 1996). Sperm preparation for IVF may, therefore, be supplemented with such antioxidant or antioxidant combinations to protect sperm membranes from ROS attack during in vitro fertilization programmes. Our present finding regarding to ROS trapping capacity of trolox in the medium is in agreement to Cao and Cutler (1993) who demonstrated that the net hydroxyl radical absorbing ability of trolox increased with increasing concentration, but only when the concentration was in the range of 0.1-20 \( \mu \)M. At higher concentration (20-40 \( \mu \)M), the hydroxyl radical absorbing ability actually decreased, and at final concentration of more than 1000 \( \mu \)M, trolox acted as an oxidation promoter (pro-oxidant) rather than an antioxidant (Cao and Cutler, 1993). The mechanism involved might be the reaction of trolox with hydroxyl radicals, which may cause the formation of additional detrimental radicals (Cao and Cutler, 1993). Donnelly and his colleagues (1999) had shown that supplementation of sperm preparations from normozoospermic and asthenozoospermic individuals with trolox and ascorbate, either singly or in combination, was not beneficial to sperm functions, particularly motility. On the other hand, Suleiman et al. (1996) reported that vitamin E treatment improved sperm motility in asthenozoospermic men. Eleven of 52 spouses in the treatment group became pregnant during the 6-month treatment period. This discrepancy in the literature about vitamin E and its analog, trolox, in improving the altered physiological processes in sperm cells might be attributed to the differences
between conditions, supplementation route and/or concentration used in their experimentations.

Trolox as an aqueous analogue of α-tocopherol (lacking phytol chain) is able to move partially into the membrane lipid bilayer to quench the free radical, and other peroxidized substances. Each molecule of trolox can trap two peroxyl radicals when oxidation is initiated in the lipid phase (Barclay and Vinqvist, 1994). This ability reflects the greater hydrophobic character of this molecule (Doba et al., 1985). Brezezinska-Slebodzinska et al. (1995) reported the protective effect of trolox against lipid peroxidation in boar seminal plasma. In this report, the ferrous-ascorbate induced TBARS production was found to be inhibited by about 62% through the trolox effect, as compared with 57% inhibition by reduced glutathione. They showed that, trolox protective efficacy was the highest at the concentration of 20 μM. These authors have suggested a scavenging action on part of trolox on the lipid peroxyl (ROO•) and alkoxyl (RO•) radicals. It may be inferred from the present results that addition of trolox to the spermatozoal samples brings about a depression in the TBARS production by either acting as a chain-breaking antioxidant or by scavenging the lipid peroxyl and alkoxyl radicals in the samples. It has been shown that trolox at concentration of 40μM protected the LDL oxidation significantly by reducing TBARS and conjugated diene production by about 43 and 80%, respectively (Lee, 2000). The author has also reported that the relative peroxyl radical absorbing capacity of trolox against uric acid and ascorbate was found to be 1: 0.86: 0.47 on a molar basis. In 1999, Albertini and Abuja showed that in the model of metal-dependent oxidation of LDL system, the effect of trolox changed from anti-oxidant to pro-oxidant suggesting that Cu²⁺/Cu⁺ redox cycling is involved in pro-oxidant mechanism of trolox in medium. Different data in literature demonstrate that antioxidant efficacy in one set of experimental conditions may not necessarily translate into a similar degree of protection in another set of conditions where lipophilicity could be a variable to the antioxidants (Massaeli et al., 1999).
There has been little attention to the possible impact of use of nicotine and caffeine on the membrane integrity and related processes in mammalian spermatozoa. From the concentration ranges of these drugs, three concentrations of caffeine (5, 7 & 9 mM) and two concentrations of nicotine (0.5 & 1 mM) were selected. The concentration of 7 mM is the optimal dose of caffeine which is applied in many parts of the world to stimulate the poor sperm samples. The selected concentrations of nicotine (0.5 & 1 mM) are the amounts estimated to approximate residual concentration of nicotine in the testes of heavy smokers (Pekarsky et al., 1995; Reddy et al., 1995). Repeatedly, selected concentrations of nicotine showed an elevation in the levels of MDA (p <0.01 & p<0.001, respectively) and in case of caffeine it was marginally elevated (p>0.05, p<0.05 & p<0.05, respectively). Nicotine is a major harmful component of cigarette smoke and mimics most of the deleterious impacts of cigarette smoke in human body (Kavitharaj and Vijayammal, 1999). The presence of nictotinic cholinergic receptors on the surface of spermatogenic cells as well as spermatozoa is demonstrated, which are able to inhibit biosynthesis of androgens in the testes (Kasson and Hsueh, 1985). Smoking may also induce higher proportions of genetically defective sperm cells (Claxton et al., 1989). It has been substantiated by Rubes et al. (1998) that cigarette smoking among teenagers was associated with an increase in disomic spermatozoa and an abrogation in specific aspects of semen quality. Such effects may alter the male fertility and may increase future chances of fathering offspring with aneuploidy syndromes. Smokers have a 13-17% reduction in sperm concentration compared with non-smokers (Vine et al., 1994; Rubes et al., 1998). The incidence of round-headed fraction of spermatozoa in smokers is associated with an increased risk of male infertility (Evans et al., 1981; Rubes et al., 1998). Cope et al. (1998) (unpublished data cited in the letters to the Editor, Hum Reprod; 13: 777-779) demonstrated that the effect of cigarette smoking on sperm motility are caused by ROS generated from leukocyte contamination of the semen and argued that these effects can
be inhibited by antioxidants. The use of free radical scavengers showed that
those such effects were due to the presence of hydrogen peroxide. Other
investigators substantiated that exposure to cigarette smoke is associated with
increased lipoperoxidation as judged by elevated lipoprotein oxidation (Frei et
al., 1991; Scheffler et al., 1992; Steinberg and Chait, 1998), and increased
concentration of circulating F₂-isoprostanes, an oxidative byproduct of
arachidonic acid (Morrow et al., 1995). Antioxidant alterations in case of
smoking have been reported by the work carried out by Smith and Hodges
(1987), in which smoking resulted in a depletion of antioxidant vitamins,
particularly ascorbate. Results from Durak et al. (1999) also suggested that
antioxidant potential is markedly lowered and TBARS level is higher in the
blood plasma samples from smokers compared with those of non-smokers.
They observed that smoking did not register any impairment in the enzymatic
antioxidant defense system like SOD and GPx in the erythrocytes, possibly
these cells have a potent and extensive antioxidant defense capacity. Smoking
is associated with an increase in strand breaks in the sperm DNA structure as
a result of oxidative stress (Potts et al., 1999). In view of these findings, it can
be speculated that smokers with marginal semen quality may be pushed into
the infertile range.

Nicotine caused an increased chemiluminescence and LPO formed 3.3-
fold in the rat oesophageal mucosa in a dose and time dependent manner
which can be inhibited by addition of SOD and catalase to the assay mixture
(Wetscher et al., 1995b). It may be considered that tobacco stimulates the
formation of ROS in oesophageal mucosa which consequently induced cell
damage. It has also been reported that metabolites of nicotine could induce
DNA single-strand breaks, which could be lessened by free radical scavengers
(Weitberg and Corvese, 1993). Wetscher et al. (1995b) substantiated that
nicotine in the absence of oesophageal mucosa does not increase luminescence
in the assay mixture which indicates that the registered enhancement of
chemiluminescence in medium was due to the oxidative stress caused by
nicotine and not due to the interaction between luminol and nicotine. These authors also demonstrated that nicotine induced the production of $\text{O}_2^-$ in media whereas $\text{H}_2\text{O}_2$ possibly was of lesser importance. In addition, it was seen that nicotine in the concentration found in the saliva of smokers caused a severe oxidative stress to rat oesophageal mucosal samples. The same result was also reported for human pancreatic tissue and oesophageal biopsies (Wetscher et al., 1995a; 1995c). In human pancreatic cells incubated with nicotine, it was seen that $\text{H}_2\text{O}_2$ was produced in a similar amount as $\text{O}_2^-$, but its toxic effect on pancreatic cell membranes may be less pronounced than that of $\text{O}_2^-$ because its depression by catalase did not significantly reduced the rate of TBARS formation (Wetscher et al., 1995a).

A positive relationship in the number of retrieved oocytes in the female patients undergoing assisted conception in relation to smoking was analyzed (Pattinson et al., 1991; EL-Nemr et al., 1998). Sharara et al. (1994) reported on a clinically detectable decreased ovarian response with increasing age of 12% of smokers, compared with 5% in non-smokers. The binding of nicotine to nuclear and cytoplasmic proteins of follicles could affect the developmental potential of maturating follicles and could lead to perturbations in meiotic maturation of oocytes (Racowsky and Kaufman, 1989; Racowsky et al., 1992). In a recent attempt, it has been demonstrated that nicotine at a 10mM concentration substantially decreased the glutathione levels (46%) and increased (3-fold) the MDA formation in the Chinese hamster ovary (CHO) cells. Simultaneously, an elevation in the lactate dehydrogenase (LDH) level was registered in the assay mixture with nicotine incubation which indicate clearly that cellular membrane integrity was damaged and disrupted (Yildiz et al., 1999). Further, the decrease in LDH activities in presence of free radical scavenging enzymes like SOD and catalase (CAT) suggested that membrane damage might be due to free radical generation.

To avoid a confounding age effect, a study in young healthy teenagers, found a trend towards poorer semen quality among smokers in association
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with cotinine in the urine, a major metabolite of nicotine (Rubes et al., 1998). Using the outcomes of the zona-free hamster oocyte penetration test, hypo-osmotic swelling test and comet assay, Sofikitis et al. (1995) showed a detrimental effect of male smoking in semen quality and its parameters like sperm concentration and motility, the results of which were lower in heavy smokers (>20 cigarette per day) than in non-smokers. Cigarette smoking is able to increase the number of diploid and/or triploid gametes in female and male individuals by binding of tobacco alkaloids, like nicotine to tubulin, a protein in the tubules of meiotic spindles which leads to disruption (Tarin et al., 1991; Zenzes et al., 1995; Rubes et al., 1998). In spermatozoa, Sun et al. (1997) reported higher proportions of oxidant radical-induced DNA fragmentations in male smokers in assisted conception, compared with the non-smokers.

Furthermore, it has been reported that inhibition of oxygen radicals with SOD could restore impaired endothelium-dependent reactivity of arterioles during both acute and chronic influence of nicotine (Mayhan and sharpe, 1998, 1999). The authors suggested that nicotine via free radical synthesis or release may cause an impairment in the vessels. The study carried out by Motoyama et al. (1997) showed that impaired endothelium–dependent vasodilation observed in chronic smokers could be restored by acute treatment with ascorbate as an antioxidant. Thus it appears that ROS plays a paramount role in impaired reactivity of large conduit vessels in chronic smokers. Another report came from Dubick and Keen (1991) which substantiated that infusion of 50 mg of nicotine over a 21 days period, caused a sharp lower levels of GSH and a decreased activity for SOD in rat liver suggesting a oxidative stress in the tissue. Free radical-dependent mechanism may play an important role in the testicular toxicity of environmental chemicals like cigarette smoke (peltola et al., 1994).

To our knowledge, little information exists on the nicotine-induced biochemical alteration during oxidative stress and subsequently LPO process.
The exact mechanism/s that mediate the effect of nicotine on the spermatozoal membrane integrity and lipoperoxidation cascade are not yet fully delineated. However, in view of the cited literature, it can be speculated that nicotine could act as a potent oxidant to different type of cells. The weight of the evidence indicates that nicotine via formation of \( \text{O}_2^- \) and to a lesser extent of \( \text{H}_2\text{O}_2 \) imposed a severe lipid peroxidation on biomembranes which was inhibited by antioxidant enzymes, as evidenced by a decrease in chemiluminescence in the medium. The existence of disruption in membrane structure is determined by leakage of LDH into surrounding environment, as a result of lipoperoxidation. The second important finding was binding of nicotine to nuclear and cytoplasmic proteins which led to cellular perturbances.

Caffeine as a methylxanthine has been reported at a highest concentration of 5 mM adversely affects the fertilization rate (38%) as compared with the control (78%). Embryonic development was also retarded and completely inhibited with caffeine (Imoedemhe et al., 1992). These authors suggested that while a definite improvement in motility may occur when spermatozoa are exposed to caffeine this improvement did not translate into enhanced fertilizing ability and subsequent embryonic development. The possible involvement of caffeine in the induction of lipoperoxidation in a sperm model system is evidenced by showing that caffeine (10 mM) increased the rotational mobility of thiol-containing proteins in a defined micellar microenvironment which was associated with inhibition of SOD activity and an augmentation in superoxide anion radical generation (Sinha et al., 1993). One of the main manifestation of these alterations to sperm cells could be considered as plasma membrane damage, probably as a consequence of LPO induction. A recent study (Jafari and Rabbani, 2000) also registered that caffeine at concentrations 5-20 mM caused a sharp increase in the superoxide anion formation and DNA degradation in alveolar macrophages from rat lung. They suggested that caffeine at moderate and high concentrations can induce
the apoptosis in rat alveolar macrophage culture. In *in vivo* system, this effect depends on the kind of cell populations and tissues exposed to caffeine. The results could be different as under different circumstances reported that the dietary maternal intake of caffeine for 22 days (2 mg / 100 mg body weight) could lead to a drop in the activity of SOD in liver, whereas recorded an increase in the SOD activity of heart of pups (Rossowska *et al.*, 1995). They suggested that caffeine was responsible to form H$_2$O$_2$ in the tissue as an oxidant but controlling the extent of damages totally depended on the antioxidant content in the respective tissues. Furthermore, the depletion of vitamin E in caffeine – induced fatty liver in rats might contribute to the induction of LPO which resulted in an increased peroxidability of tissue.

In another attempt, it has been established that following giving a dose of 100 mg /kg of caffeine to rats, the hepatic GSH levels were decreased by about 22.50% of the control values. Subtracting the glutathione molecules from media would be followed with a subsequent induction of LPO (Farag and Abdel – Meguid, 1994). Kamat *et al.* (2000) reported that under anoxic conditions (have no saturation with O$_2$), caffeine could potentiate the TBARS formation in radiation-induced lipoperoxidation by decreasing the restorative reactions between radiation-induced free radicals such as RO$_2^-$ in membranes.

It has also been shown that another methylxanthine namely pentoxifylline (PTF) has some deleterious impacts on spermatozoa. Gavella and Lipova (1994) demonstrated that PTF might further augment the ferrous ion-stimulated decomposition of pre-accumulation of lipid hydroperoxides in the sperm plasma membrane and promote MDA generation in a TBA assay. It can be concluded that the stimulatory effect of PTF on iron-induced lipid peroxidation may have an adverse effect on the quality of spermatozoal suspensions prepared for IVF.

Membrane of normal spermatozoa have high fluidity of the lipophilic domains which are corresponded to lipid matrices with strong inter-molecular affinity within each matrix. At the same time, these matrices allow rotational
motion of the molecules within their confinements. Nivsarkar et al. (1996) reported a post-treatment increase in the fluidity profiles after exposure of spermatozoa from oligospermic individuals to PTF. Oligospermic samples show low membrane fluidity. PTF could inhibit the SOD activity which was due to an increased rotational motion of the membrane lipids and a weakened lipid-protein lattice structure (Nivsarkar et al., 1996). The resultant $\mathrm{O}_2^-$ accumulation induces a lipid-phase transition and altered molecular mobility in membrane framework. Based upon these findings, it can be concluded that PTF or similar methylxanthines modulate the sperm membrane conformation by increasing the membrane mobility which is in response to an elevation in peroxidation rate in the membrane fatty acid chains by increased $\mathrm{O}_2^-$ through an oxyradical mechanism augmented by those agents.

Recent *in vivo* data have indicated that the xanthine oxidase (XO) system participate in the metabolism of methylxanthines like caffeine in the body. Ratio of caffeine metabolites excreted in the urine after a caffeine challenge is considered to be an indicative of the total XO activity in human (Boda et al., 1999). In addition, it has been suggested that the sperm damage can be induced *in vitro* by addition of methylxanthines to medium containing xanthine-oxidase system (Lenzi et al., 2000). It resulted in the formation of high quantities of hydrogen peroxide as the most stable intermediate of $\mathrm{O}_2$ reduction which is produced by univalent reduction of molecular oxygen and crosses biomembranes almost as freely as water. In view of these findings, it can be speculated that prolonged or excessive ROS in spermatozoal samples after addition of certain concentration of caffeine is due to a possible induction of XO-like activity in the seminal plasma or sperm plasma membrane which leads to chain reactions of lipoperoxidation.

Taken together, the following proposed mechanisms may be contributed to pro-oxidant activity of caffeine/methylxanthines when added to the spermatozoal suspensions: i) Excessive release of restored and pre-existing hydroperoxide radicals from membranes. In turn, these radicals reattack the
membranes and lead to the formation of new radicals in media (Shi et al., 1991; Kamat et al., 2000). ii) Inhibition of SOD activity and subsequent increase in $O_2^-$ formation which imposed a severe peroxidation challenge to membrane components as well as inhibition of antioxidant enzymes like GPx and CAT (Sinha et al., 1993; Rossowska et al., 1995 & Nivsarkar et al., 1996). iii) Possible stimulation of a xanthine oxido-reductase–like enzymatic system by caffeine in the spermatozoal samples being able to generate more hydrogen peroxide molecules in assay mixture which leads to lipoperoxidation induction and inhibition of antioxidant enzymes.

Besides the lipotoxicity impact of caffeine, some reports have emphasized on the mutagenic activity of caffeine, in which caffeine could inhibit the DNA synthesis and repair, with higher affinity for DNA damaged regions and denaturated points so that repair enzymes were left ineffective (Tolmach et al., 1977; Lau and Pardee, 1982 & Selby and Sancar, 1990).

The ROS are basically chemical species, endowed with an unpaired electron, which react promptly with both free radicals as well as non-radical molecules, thus triggering the spread of sequence of radical reactions. The levels of peroxidable substances, such as PUFA, and the levels and the activity of the free radical scavenger systems generally regulate cellular homeostasis. Therefore, an oxidative stress can be defined as any disturbance of the balance between anti-oxidants and pro-oxidants, with the former prevailing (Lenzi et al., 2000). Due to the loss of most of its cytoplasm in maturation, it leaves sperm cell at a significant disadvantage lacking the endogenous repair mechanisms against oxidative insult, but at the same time they are protected by seminal plasma which contains an abundance of antioxidant enzymes such as SOD (superoxide dismutase), CAT (catalase) and GPx (glutathione peroxidase) which remove key ROS such as $O_2^-$ and $H_2O_2$, and scavengers like albumin and taurin. Seminal plasma also contains crucial chain-breaking antioxidants such as urates, ascorbate and thiol groups (Donnelly et al., 1999). Antioxidants, in general, are compounds which dispose, scavenge, and
suppress the formation of ROS, or oppose their actions. Seminal plasma from infertile men showed that there has been a significantly lower antioxidant capacity, at least in part, in a reduced ascorbate concentration (Lewis, et al., 1995, 1997).

Depriving sperm of the antioxidant protection available in seminal plasma leads to damage to DNA, and the protection can be provided by supplementing sperm preparation media with antioxidants such as ascorbate and α-tocopherol even if no free radical activity can be detected (Hughes et al., 1998).

In the present work, one concentration of each antioxidant was selected such as, 1mM ascorbate, 5mM glutathione, and 0.02 mM trolox according to the most effective dose of each compound, and used in single and/or in combination to counter the nicotine and caffeine-induced changes in spermatozoal samples throughout the present work. Depleted ascorbate levels observed in heavy smokers have been associated with decreased sperm motility (Shaarawy and Mahmoud, 1982). In addition, preimplantation of mouse embryos have benefited from the addition of antioxidants and iron chelators to culture media, presumably by overcoming the adverse effects of free radicals on embryonic development (Nasr-Esfahani et al., 1990). Therefore, there was reason to expect that antioxidant supplementation would benefit sperm functions and possible related alterations.

Our results regarding to the effectiveness of antioxidants on the spontaneous or nicotine and caffeine-induced lipid peroxidation in spermatozoal samples show that trolox (0.02 mM) was more effective singly than ascorbate (AA) and glutathione in lowering the levels of TBARS in assay mixture. Therefore, trolox could exhibit an excellent protective role against inducing LPO by nicotine and caffeine as compared to AA and GSH. Interestingly, it has been recently reported that trolox could bind to some proteins like human serum albumin and inhibit structural damage of these proteins and act as a very potent protector following oxidative damage (Salvi.
The results of previous experimental studies confirmed an efficient free radical absorbing capacity for trolox. Trolox also traps effectively the membrane peroxyl radicals (Doba et al., 1985). It could prevent oxidative reactions initiated upon hemoproteins by hydrogen peroxide, involving tyrosyl radicals in the sperm whale myoglobin (Giulivi and Cadenas, 1993). In in vitro model, trolox was also demonstrated to have desirable scavenging ability to protect the tissues against lipoperoxidation (Casini et al., 1985; cited in Doba et al., 1985). Our observations suggest that supplementation in vitro with antioxidants, trolox, ascorbate and GSH separately has beneficial effect for spermatozoal membrane integrity towards stability of cellular framework. Recently, there has been much debate regarding the potential advantage of antioxidant therapy in improving male infertility and increase in chance of a successful conception (Ford and Whittington, 1998; Tarin et al., 1998; Albertini and Abuja, 1999; Donnelly et al., 1999; Upston et al., 1999). However, it is recognized that this therapy may be a double-edged sword, with considerable undesirable effects if a safety threshold concentration is surpassed (Tarin et al., 1998). For example, excessive levels of ascorbate are thought to be associated with the inhibition of ovarian steroidogenesis (Levine and Morita, 1985), a decline in fertility (Igarashi, 1977), and an increased likelihood of abortion (Pintauro and Bergan, 1982). It has also been stated that although the role of antioxidants in sperm function is fascinating, further research is required before we can be optimistic about potential role for antioxidants in the treatment of male infertility (Martin-Du Pan and Sakkas, 1988).

In order to examine the effectiveness of combined concentration of antioxidants on lipid peroxidation, different combinations of antioxidants have been supplemented in the assay mixture. The combined concentration of glutathione and trolox has proved to produce the best effect in lowering the spontaneous lipid peroxidation (72.60%) of untreated-unsupplemented sample, whereas other combinations namely ascorbate+trolox and
ascorbate+glutathione were able to reduce the MDA levels by about 67.80 and 67.47%, respectively. On the other hand, in nicotine-treated samples the combination of ascorbate+trolox and in caffeine-treated samples the combination of glutathione+trolox were substantiated to produce the best results. As cited earlier, antioxidant defence system works in an integrated fashion in sperm cells to minimize the oxidative damage. Besides the activity of antioxidant enzymes like SOD and GPx and/or CAT, α-tocopherol is present as a chain-breaking antioxidant in membranes and available to eliminate the generated ROS. Its oxidation produces the tocopheryl radicals, which can then be reduced by ascorbate molecules. The oxidation of ascorbate in turn, gives rise to ascorbyl radical, which can be reduced and quenched by glutathione, producing the thyl (GS) radicals (are not as rapid as oxidative attack at PUFA of membranes and therefore are not very harmful to cells) and oxidized GSH, which can be regenerated by glutathione reductase (GRD) activity (Poli et al., 1993). As a consequence, the whole system has to work simultaneously and an alteration of one of the components, for example inhibition of antioxidant enzymes and/or presence of excessive amount of each antioxidants or their interactions with metal ions, can lead to potentially damaging accumulation of ROS.

Seminal plasma must be regarded as an excellent nutritive and protective medium for sperm cells and possess higher concentrations of antioxidants than other biological fluids or blood serum. It becomes aggressive only when pathologies alter its anti/pro-oxidant equilibrium or when the sperm membrane is more fragile or weaker after in vitro manipulations (Lenzi et al., 2000).

High levels of antioxidants have been positively correlated with the semen quality (Fraga et al., 1991; 1996; Kobayashi et al., 1991; Suleiman et al., 1996). The epididymis is a rich source of antioxidant enzymes that scavenge any excess of reactive oxygen metabolites released by the sperm cells during epididymal transit (Aitken and Vernet, 1998). Combinations of antioxidants
can therefore be used to treat the sperm abnormality cases, as they can restore the physiological constitution of PUFA in the cell membrane. It is recommended that spermatozoal samples can be treated with any antioxidant/s prior to *in vitro* fertilization. This procedure can also prove to be beneficial after cryopreservation, a condition in which increase of peroxidative damage has been cited (Royere *et al.*, 1996).

In the rare cases and under certain circumstances, antioxidants exert a pro-oxidant ability to promote the peroxidative damage. A pro-oxidant effect of \( \alpha \)-tocopherol was found under mild oxidative conditions in highly diluted (150-fold) plasma and in isolated LDL (Kontush *et al.*, 1996). It was suggested that \( \alpha \)-tocopherol may involve into peroxidation, when the co-antioxidants in combination are exhausted under certain conditions. Albertini and Abuja (1999) substantiated that presence of other antioxidants in a model of metal-dependent oxidation of lipoproteins could change the ability of trolox from pro-oxidant to an anti-oxidant form.

A combined oral antioxidant dose containing vitamins E and A plus N-acetylcysteine on sperm biology in the subfertile men resulted in significant reduction in ROS formation; increased sperm concentration in oligospermic men; a decrease in the level of oxidized DNA and finally an increase in the PUFA concentrations and membrane fluidity. The results came from Leedle and Aust (1990) indicating that glutathione can be used to maintain vitamin E concentration, thereby lowering the microsomal lipoperoxidation process in experimental conditions.

It is reported that the synergistic interaction between vitamin E and C caused a depression in lead-induced ROS generation and protected the spermatozoa from loss of motility and oocyte penetration capacity (Hsu *et al.*, 1998). Co-antioxidant activity or synergism between vitamin E and C has been observed to delay the MDA production during ferrous-catalysed peroxidation of rat liver microsomal and phospholipid liposomes (Leung *et al.*, 1981; cited in Doba *et al.*, 1985). In another report, it has been substantiated that for
peroxidation initiated in the bilayer region, glutathione is comparatively inefficient in its interaction to preserve either vitamin E in the lipid phase or trolox in the water phase (Barclay, 1988).

In order to evaluate the impact of iron containing compounds on spontaneous as well as drug-treated peroxidation in membrane lipids, the sperm samples were subjected to 0.2 mM concentration of ferrous sulphate (FeSO₄), along with antioxidant supplementation. It was proven that, addition of ferrous to the untreated samples caused a very sharp elevation in the extent of spontaneous LPO production in assay mixture by about 59.4% (p<0.001).

The resultant iron-catalysed lipoperoxidation was found to be extended upon addition of different concentrations of nicotine and caffeine to media. Elevation in the levels of MDA in treatment with the highest concentration of nicotine was 115.7% and in case of caffeine, it was even raised by about 125.6%. It can be suggested that the emerged complexes of Fe²⁺ with nicotine and/or caffeine may act as a more potent oxidant which is the reason behind the propagation of lipoperoxidation throughout the membrane framework. This linkage and resultant peroxidative impact was stronger in case of caffeine than that of nicotine. At least for nicotine, a very plausible mechanism of action may be the ability of this compound to bind directly to Fe³⁺, probably via the pyridine nitrogen (Goerig et al., 1992; Cited in Linert et al., 1999). It can be said that this complex might be responsible for the nicotine impact in presence of ferrous ion in the medium which resulted in an increased rate of MDA formation.

Normally, the lipid hydroperoxides (Lipid-Ο₂H) tend to accumulate and stabilize in plasma membrane, unless a transition metal, like ferrous is added to the cell suspension, it causes a sudden acceleration of lipid peroxidation with formation of alkoxyl and peroxyl radicals and results in the extension of LPO process (Halliwel and Gutteridge, 1984 & Sharma and Agarwal, 1996):

\[
\text{Lipid -O}_2\text{H} + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{Lipid-O'} \text{(Alkoxyl)}.
\]

\[
\text{Lipid -O}_2\text{H} + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{H}^+ + \text{Lipid-O}_2 \text{(Peroxyl)}.
\]
Discussion

It is also of great interest to mention that another existing mechanism to iron-catalysed lipid peroxidation is the reactivity of mixtures of iron (II) with hydrogen peroxide in media which results in the formation of hydroxyl radical (OH) capable of extensive damage to the cell organelles. This is known as Fenton’s reagent after Fenton (1894), which is of particular importance in iron-dependent OH· radical damages:

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \]

In view of the above findings it can be speculated that complexes between Fe\(^{2+}\) and nicotine and/or caffeine could release more amounts of alkoxy and peroxyl radicals from the pre-existing lipid hydroperoxides in the plasma as well as organellar membranes in the cell.

In connection to harmful effects of iron, it was reported that iron has an ability to introduce the harmful hydroxyl substituents into organic substrates which in turn, leads to an inactivation following a polymerization process (Jameson and Linert, 2000).

Our results have shown that antioxidant supplementation caused a sharp decline in the levels of MDA except in the case of ascorbate (alone), which resulted in a further elevation in the rate of MDA formation in all the cases, e.g. 89.2% (p<0.001) in untreated – Fe\(^{3+}\) supplemented series. It may be suggested that a synergistic effect was established between ascorbate and ferrous as iron-ascorbate complex which functioned as a LPO promoter. A different mechanism for lipoperoxidation has been suggested in suspension of human spermatozoa observed in the presence of ferrous ions (Aitken et al., 1993). It is the catalytic decomposition of lipid hydroperoxides pre-existing in the spermatozoa and subsequent propagation of peroxidation chain reaction through creation of alkoxy and peroxyl radicals (Aitken et al., 1993). Guerin et al. (2001) also emphasized on this point that, in the presence of transition metals, ascorbate shows a pro-oxidative activity by converting ferric into ferrous ions, thereby catalyzing the Fenton reaction.
Ascorbate also has the ability to promote the release of transition metals from proteins, to redox iron and copper to form oxygen radicals, and therefore, to act as a pro-oxidative agent when added in isolation (Gutteridge, 1994).

In the present study, it was also registered that trolox when added singly, was the best to lessen the extent of ferrous-induced and iron-ascorbate induced lipid peroxidation in the assay mixture. Glutathione was not considered as a match for trolox to depress the level of MDA in medium, although it could also inhibit markedly the rate of LPO in ferrous-treated samples. Interestingly, in the nicotine+ferrous-treated samples when supplemented with combination of ascorbate+trolox and/or glutathione+trolox, it was found that the level of MDA was even lower than the respective drug-treated spermatozoal samples. Here again, using the combination of glutathione+trolox resulted in a lower level of MDA in caffeine+ferrous-induced LPO in human spermatozoal samples.

In order to examine the dose response of the promoter system, and calculate the kinetic parameters of lipid peroxidation the experiments were performed with different promoter concentrations. All of these had same combination ratio of 1:5, ferrous sulphate: ascorbic acid, respectively. This ratio (1:5) has been suggested to be effective by Aitken et al. (1989) and therefore employed in the present study. Subjecting the human spermatozoal suspension to increasing concentrations of ferrous sulphate – ascorbic acid (iron-ascorbate) complex resulted in an increased lipid peroxidation. The data has been treated according to Michaelis-Menten enzyme kinetics by extrapolating the combined promoter ratio of the individual combination as substrate concentration [S] and treating the observed amount of MDA produced, equivalent to initial velocity (v_0). The kinetic parameters k_m and V_max were calculated from the Lineweaver-Burk double reciprocal plot. The experimental data was also subjected to regression analysis.
A linear increase, positively correlated with substrate concentration was observed in the untreated-unsupplemented series (r=+0.985). The values of correlation coefficient (r) in 0.5 and 1 mM nicotine concentrations, were calculated to be +0.971 and +0.967 and for 5,7 & 9 mM caffeine concentrations to be +0.970, +0.970 and +0.991, respectively.

Nicotine and caffeine were noted to augment the process of lipid peroxidation while the ascorbate has an ability to promote the iron redox cycle in biological fluids, which resulted in the formation of more toxic substances endangering the cells (Gutteridge, 1994). Using the iron-ascorbate LPO promter system, Kamat and Devasagayam (2000) put seal on this point that this widely used oxidant generating system in vitro is responsible to produce a number of deleterious radical species in membranes to impose a severe oxidative stress. It is still unclear why such association between nicotine and caffeine with iron-ascorbate system exists in increasing the level of MDA.

In the present study, two antioxidants namely glutathione and trolox when used singly and/or in combination, during the present study revealed that after supplementation of antioxidants to control and the drug-treated spermatozoal samples the maximum amount of malonyldialdehyde production (V_max) was alleviated. The best effect was produced by the combined concentration of glutathion+trolox in lowering the rate of reaction (V_max). The k_m of reactions remained unchanged. From the present study, it may be postulated that a higher k_m and lower V_max value is favourable to the cell such that it indicates a lowered oxidative stress or in other words a depressed lipoperoxidation in assay mixture.

The present study also indicates that the lowering of MDA content by antioxidant supplementation is not brought about by its binding to the same site as that of ferrous ascorbate, neither does it modify the ferrous ascorbate binding sites. Whatever mechanism of the antioxidants might be following, the present results may prove benefiting for the sperm cells in cryopreservation.
and other techniques in assisted reproduction, protecting them against oxidative damage.

**Spermatozoal Lipid Analysis**

Mammalian cell membranes consist of lipid bilayer composed primarily of phospholipids and cholesterol. Proteins that have paramount cellular functions, such as receptors, transporters, and enzymes are embedded in the lipid bilayer interacting with dynamic state of lipids, as suggested in the fluid mosaic model of membrane structure. Lipids are also known to influence the function of certain proteins in the cell membrane.

It is known that various phospholipid classes are asymmetrically distributed in specific halves of membrane: choline-containing phospholipids namely phosphatidylcholine (PC) and sphingomyelin (SPM) predominate in the outer monolayer while amino-containing phospholipids namely phosphatidylethanolamine (PE) and phosphatidylserine (PS) dominate the inner leaflet (Bretsher, 1975; Golan et al., 1984). Malonyldialdehyde, as a by-product of lipid peroxidation process, has been shown to destabilized the lipid asymmetry (Yuli et al., 1981). The lipid asymmetry in sperm membranes is further complicated by the presence of acrosomal membrane beneath the plasma membrane. Apoptotic cells expose higher amount of PS on the exoplasmic plasma membrane leaflet, whereas in cell, that are not undergoing apoptosis PS is almost totally confined to the cytoplasmic leaflet of the plasma membrane. Furthermore, recognition of aged erythrocytes and apoptotic cells by macrophages could be related to a loss of membrane asymmetry and exposure of PS class on the exoplasmic leaflet (Krahling et al., 1999; Callahan et al., 2000). Many of the proteins in the cytosol bind to the plasma membrane through interaction with charged phospholipids which is vital to their actions for example, protein kinase C activity strongly depends upon PS concentration and presence. Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin are the major phospholipids and zwitterionic, while the acidic
phospholipids (negatively charged) are represented by phosphatidylserine and phosphatidylinositol (Stryer, 1995).

Lateral diffusion of lipids in the membrane environment may allow proteins to undergo conformational changes necessary for receptor and transport functions. Membrane-associated factors governing lipid mobility in biological membranes include cholesterol content, phospholipid composition, and protein content of the membranes. Cholesterol is one of the main lipid components of mammalian plasma membranes. It represents approximately 45% of lipids in the human erythrocyte membrane and is therefore regarded as a major determinant of rigidity, permeability for anions and osmotic fragility of the membranes (Locher et al., 1984). It also influences membrane-bound enzymes, such as Na⁺-K⁺-ATPase activity. A positive correlation between the calcium influx and the molar cholesterol/lecithin (PE) ratio of the human red blood cell membrane has been established (Locher et al., 1984). Normally, the molar ratio of cholesterol/phospholipid in erythrocyte membrane is about 0.8-0.9. Increase in the membrane viscosity occurs until the molar ratio of cholesterol to phospholipid is 2.0, but not further (Spector and Yorek, 1985).

Karnovsky et al., (1982) indicated that, most of altered cellular functions are probably caused by direct modifications in membrane lipid composition. According to this view, certain receptors, transporters, and enzymes are sensitive to the structure and physical properties of lipids with which they react. This sensitivity may involve conformational changes that affect the binding sites of receptors and transporters or the active site of enzymes. Changes in lipid framework may thus lead to altered lateral mobility of proteins resulting in functional changes.

The present study was undertaken to evaluate the impact of nicotine and caffeine on the human sperm lipid composition with/without antioxidant supplementation. Incubation of human spermatozoal homogenates with nicotine (1 mM) and caffeine (7 mM) resulted in a significant decrease in the
content of total lipids in the sperm samples, by about 37.62 & 25.36% (p<0.001 & p<0.01), respectively. These results are parallel with those obtained from measurement of lipoperoxidation in which nicotine and caffeine were considered as potent and mild oxidants, respectively.

According to Hegarty et al. (1982), cigarette smoking brings about a depression in high density lipoprotein surface phospholipids. Chronic intraperitoneal injection of nicotine in rats have also been shown to produce a significant reduction in total serum phospholipids (Bizzi et al., 1972). These polar lipids normally participate in exchange reactions with plasma membranes, serve as carriers for long chain PUFA and also play a paramount role in the normal functioning of the lipid transport/delivery system (Jackson and Gotto, 1974). In heavy smokers, it has been established that cigarette smoke by-products can move across the blood-testis barrier and exist at high concentrations in the testes which may adversely affect sperm functions (Stedman, 1986). It was also substantiated that nicotine has the ability to cross the membranes and reach the cytoplasm of ovarian cells, leading to perturbations in meiotic spindles (Racowsky et al., 1992). It is at the time of membrane crossing, that nicotine can introduce some alterations into the lipid structure of plasma membranes.

Caffeine is easily soluble in most organic solvents. This methylxanthine is enough lipophilic to traverse through the cell membranes (Kihlman, 1977; Blanchard and Sawers, 1983). Caffeine can also cross the placental cells in female individuals (Tarka, 1982). It is suggested that caffeine may enter the phospholipid bilayer and then exit from it by solvent systems. The interaction of caffeine in the lipid bilayer might cause a change and thereby the presence of caffeine brings about a change in the lipid fraction as judged by the induction of lipid peroxidation in membrane elements.

The current investigation substantiated that upon addition of nicotine and caffeine to spermatozoal homogenates a decline in the content of total phospholipids was registered by about 40.32 & 16.32% (p<0.01 & p<0.05),
respectively. Nearly, similar decline in the level of phosphatidylinositol + phosphatidylserine complex and sphingomyelin following nicotine and caffeine addition was recorded, but phosphatidylcholine + phosphatidylethanolamine complex did not show a drastic drop, so drastic so as drug-induced lipoperoxidation could not alter the content of these two phospholipid classes in the homogenate. It can be speculated that there is a good translation of altered phospholipid classes into induction of LPO cascade during drug incubations. Taken together, it is plausible that the specific modification in the phospholipid composition results from an induced lipoperoxidation process.

Sperm membranes play a very active role in sperm fertilizing capacity in sperm-oocyte cross talk. In mammalian spermatozoa, biomembrane fluidity can increase in parallel with the increase of the degree of unsaturation (associated with the fatty acyl component of phospholipids) when sperm cells pass from the caput to the cauda of epididymis. This indicates an active lipid metabolism of the spermatozoa as a complete rearrangement of the pre-existing membrane structure (Cowan et al., 1986; Hall et al., 1991). Current theories of membrane fusion suggest that membrane fluidity is a prerequisite for normal cell function and that the fluidity and flexibility of cell membranes are mainly dependent on their lipid constitution, particularly phospholipid composition (Lenzi et al., 2000). Therefore, it can be suggested that any alteration in these membrane components will lead to a failure in the sperm-oocyte communication.

Seminal plasma has a highly specialized scavenger/antioxidant system that defends the sperm membrane against lipoperoxidation, enzymatically and/or non-enzymatically. Various pathologies and systemic predisposition can lead to an antioxidant/pro-oxidant disequilibrium (Lenzi et al., 1996). Furthermore, in the in vitro studies, due to a lack of seminal plasma after washing the samples and actually a lack of natural scavengers, the sperm cells
Discussion

are endangered to attack by many of pathological states and various oxidants by which the membrane structure would be altered.

The rate of loss of fatty acids from the various phospholipid types is dependent on the type of phospholipid and medium conditions. In the membranes, phospholipase A₂ may act in peroxidative defense by excising a hydroperoxy acyl group from phospholipid and providing the hydroperoxy fatty acid product as substrate to glutathione peroxidase (Alvarez and Storey, 1995).

In the present study, it was demonstrated that the content of total cholesterol remained almost unchanged with a very little decline upon nicotine and caffeine treatments by about 8.16 & 3.26% (p>0.05), respectively. It can be speculated that nicotine and caffeine could not alter the rigidity of plasma membrane under the experimental conditions of current study. Cholesterol as a major part of plasma membrane can reduce the insertion of proteins into membrane phospholipids (Muller and Krueger, 1986). Moreover, it has been demonstrated that cholesterol can inhibit the lateral motility of protein receptors, modulating their activity and changing their conformation (Yeagle, 1989). This is also supported by the fact that removal of cholesterol triggers membrane destabilization and allows protein migration, which is the basis of capacitation (Nolan and Hammerstedt, 1997).

Cross (2000) substantiated that the phospholipid sphingomyelin in the human sperm cells can influence the rate of capacitation by slowing loss of sterols particularly cholesterol, and that exogenous sphingomyelinase accelerates capacitation by speeding the loss of sterols and by generating ceramide.

The content of free fatty acids (FFA) in the spermatozoal homogenates was found to be decreased significantly upon incubation with nicotine (46.32%, p<0.01) and caffeine (27.78%, p<0.05). Caffeine again showed that it functioned as a very mild peroxidative agent than nicotine in eliminating the detectable FFA in the spermatozoal homogenate under certain experimental
conditions. There is not much evidence regarding the role of these two agents in peroxidation process in sperm membrane. The complete maturation of the sperm cell membrane is attained after testicular lipid biosynthetic processes and after passage through the epididymis. The sperm membrane phospholipids contain a notable amount of polyunsaturated fatty acids (PUFA). The three families of PUFA are classified according to the distance of the first double bond from the methyl terminal, i.e. n-3, n-6 and n-9. Long chain PUFA in sperm cell membrane phospholipids drive from the metabolism of essential linoleic acid (C18:2 n-6) and α-linoleic acid (C18:3 n-3). These di-unsaturated fatty acids are normally assumed via the normal diet are converted into their long chain derivatives by a series of elongation and desaturation. PUFA are known as the precursors of prostaglandins and leukotrienes which are important factors in sperm motility (Lenzi et al., 2000).

Gandini et al. (1999) substantiated that, among the metabolites deriving from the desaturase activity, detectable concentrations were of C20:3, C20:4 and C22:4 (belonging to n-6 series) and of C20:5 and C22:6 (from n-3 series). Among these, n-6 PUFA represented 1/3 and n-3 series 2/3. Docosahexaenoic acid (DHA, C22:6 n-3) was found in extremely high levels in human and other mammal ejaculates with the majority occurring in the spermatozoa (Kelso et al., 1997; Conquer et al., 1999; Gandini et al., 1999). The high percentage of DHA correlates with normal morphology (Lenzi et al., 2000) and good motility (Gulaya et al., 2001) in human spermatozoa. Phospholipids which contain C22:6 n-3 are not equally distributed throughout the membranes of mammalian spermatozoa, being located mainly in the tail (Connor et al., 1998). The asthenozoospermic men have lower levels of DHA than normozoospermic men and also the ratio of n-3 to n-6 fatty acids is lower in asthenozoospermic patients (Conquer et al., 1999).

In infertile men, a drastic loss of n-3 PUFA was observed (Gulaya, 2001). Fluoride-induced lipoperoxidation caused a significant decrease in the PUFA content of rat kidney cells (Guan et al., 2000). Cerolini et al. (2000)
reported that in the boar spermatozoa during liquid storage the content of 22:6 n-3 PUFA was decreased, whereas the content of saturates, mainly 18:0 was increased significantly. The immature germ cells contain lower amount of DHA than mature sperm cells, also an inverse relationship is established between atypical sperm forms and the percentage of DHA in human spermatozoal samples (Lenzi et al., 2000a). In contrast to mammals, the phospholipids of avian spermatozoa are characterized by very high proportions of C20-22, n-6 PUFA, mainly docosatetraenoic (22:4 n-6) and arachidonic (20:4 n-6) acids. Interestingly, in duck spermatozoa there is a very high proportion of C22:6 n-3 PUFA in PS and PE fractions of membrane lipids (Surai et al., 1999).

The total phospholipid and fatty acid content of phospholipid fractions of membrane can be altered under certain conditions such as cryopreservation and aging. The total phospholipid content of spermatozoa was decreased during liquid storage, while no quantitative decline in seminal plasma. The significant decrease was drastic in phosphatidylcholine content (Douard et al., 2000). Kelso et al. (1997) reported that with increasing age there was a large decrease in the proportion of PE and PI, accompanied with an extensive reduction in the content of PUFA, arachidonic 20:4 (n-6) and docosahexaenoic 22:6 (n-3) acids. They also demonstrated that the changes in lipid composition of bull spermatozoa owing to aging were associated with a marked reduction within the seminal plasma antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD). Interestingly, it was substantiated that addition of phosphatidylcholine to immature and immotile sperm cells from the caput of human epididymus developed a good progressive motility, so that PC is strongly associated with induction of rapid motility (Haidl and Opper, 1997).

In the present investigation, the effectiveness of antioxidants either singly or in combination on alterations in lipid fractions was evaluated. It was registered that antioxidants failed to lessen the drug-induced alterations in
most of the sperm membrane lipid fractions when used in single concentration. On the other hand, combined concentrations of the antioxidants functioned satisfactorily in mopping up the free radicals from the lipid media in sperm samples. The scavenging role of antioxidants in removing the free radicals in samples treated with nicotine was more prominent than those of samples treated with caffeine. It remains to be determined however, whether this event is simply a consequence of a kind of complex between antioxidants and derivatives of these agents or not. Furthermore, in most of drug-treated sperm suspensions, trolox alone and also in combination with glutathione exerted an effective scavenging role in lessening the peroxidative damages in media. As mentioned earlier, antioxidant usage could decrease the extent of lipoperoxidation in biomembranes (Meister and Anderson, 1983; Aitken and Clarkson, 1988; Den Boer et al., 1990; Cao and Cutler, 1993; Suleiman et al., 1996; Hsu et al., 1998; Albertini and Abuja, 1999; Comhaire et al., 2000; Donnelly et al., 2000; Lee, 2000; Lenzi et al., 2000). It can be taken into account that any decline in the extension of lipoperoxidation by the function of antioxidants may be translated into an early improvement in peroxidation of lipid components of sperm membranes.

Functioning of the cell is greatly affected by alterations in the lipid microenvironments of the membrane and also the PUFA moiety of membrane lipids is readily oxidized by free radicals. Whatever is the molecular mechanism of nicotine and caffeine interaction in the spermatozoal membranes, it may be assumed that the enzyme activity is a lipid sensitive process and this process is inactivated in a situation where the membrane lipid microenvironment is modified beyond a reasonable limit. Taken together, these types of alterations in sperm membrane could bring about a marked change in human fertility.
**Glutathione metabolism and \( \gamma \)-glutamyl cycle**

The ultimate goal of a spermatozoon is the successful fertilization of ovum resulting in normal conception. Infertility has been a major medical and social concern throughout the world and defective and/or poor sperm function is the most prevalent cause of male infertility (Hull et al., 1985). Many environmental, physiological, and genetic factors have been implicated in the poor sperm function and resultant infertility.

Free radical-induced oxidative damage to spermatozoa is one of the deleterious condition which is recently gaining considerable attention for the poor semen quality (Sikka, 1996). One of the most prevalent non-enzymatic antioxidant defence systems against free radical insult in the cells is found to be reduced glutathione (GSH). Studies have been reported in the literature on the role of glutathione as an effective antioxidant and related \( \gamma \)-glutamyl compounds in removing free radicals from the media (Meister and Tate, 1976; Meister, 1983; Zigler, 1985; Sikka, 1996; Smith et al., 1996; Lenzi et al., 2000).

The intracellular synthesis of GSH and its utilization are linked by the \( \gamma \)-glutamyl cycle, a series of enzyme-catalyzed reactions. In the present study, an attempt has been made to identify the nicotine and caffeine-induced changes in glutathione metabolism and the components of \( \gamma \)-glutamyl cycle and considering the effectiveness of antioxidants. The study first targets the measurement of total sulfhydryl (\(-SH\)) groups, GSH content as non-protein bound \(-SH\) groups, and GSSG content as protein-bond \(-SH\) groups under normal and oxidative stress conditions in human spermatozoa.

The total thiol content in the nicotine (0.5 & 1 mM) and caffeine (5, 7 & 9 mM)-treated sperm suspensions remained non-significantly (\( p>0.05 \)) altered, except in the case of 9mM caffeine treatment where it was a significant increase (22.40%, \( p<0.05 \)). Antioxidant supplementation did not register significant alteration in most of the samples except in 1mM nicotine-treated ones in which addition of trolox (0.02mM) and/or combined concentrations of ascorbic acid (1mM) and trolox resulted in an elevation in total thiol group.
(p<0.05) as compared to the control data. On the other hand, the reduced glutathione (GSH) content was recorded to be slightly decreased in nicotine-treated samples (p>0.05) while and raised in caffeine-treated series (p>0.05). However, the oxidized glutathione (GSSG) level was considerably elevated in nicotine treatments than that of caffeine. Addition of antioxidants could not change the level of GSH, as compared to the respective drug-treated samples.

Antioxidant supplementation, ascorbic acid and trolox (singly and/or in combination) caused a drop in the level of GSSG, showing that the used antioxidants could scavenge the free radicals from the samples, resulting in a lower rate of conversion of GSH to GSSG and eliminating the oxidative stress. Taken together, this may be related to the lowered TBARS formation under these conditions. Amongst these, the combination of AA and trolox had a paramount effect to lessen the GSSG content in different groups. Since under antioxidant supplementation the extent of lipoperoxidation was decreased, the need of conversion of GSH to GSSG was also lowered. Also a level of membrane sulfhydryl groups has to be maintained so as to ensure the required membrane fluidity that is a prerequisite for normal sperm function (Nivsarkar et al., 1998).

During lipoperoxidation (LPO), proteins are exposed to a wide range of free radical species generated from the lipids, which are capable of oxidizing protein sulfhydryl groups, thus promoting the formation of disulfide bridges, and even inducing protein fragmentation. The loss of protein –SH groups has been implicated in the loss of cell viability in several experimental conditions (Thomas et al., 1987). It was also revealed by the work of Fulceri et al. (1990) that the LPO-derived aldehydes originate and accumulate in the lipid domain of the membranes. The possibility, therefore, exists that lipid peroxidation through the formation of reactive aldehydes may cause a selective loss of protein thiols in cellular membranes (Pompella et al., 1991). Most recently, Ehrhart and Zeevalk (2001) revealed that glutathione (GSH) under a reversible nature of formation can bind to the proteins and gain protein-GSH
mixed disulfide formation which under these circumstances may play a
paramount protective role against oxidative stress.

The presence of oxidized thiol groups in structure of erythrocyte
proteins leads to protein dysfunction and inactivation (Snyder et al., 1988 &
Wang et al., 1999). In this connection, Sinha et al. (1991) showed that in
oligospermic men there is a quantitative reduction in the −SH groups in the
sperm head membrane compared to that of normospermic men. Interestingly,
Mamamoto et al. (1996) substantiated that ROS could block sperm-oocyte
fusion via oxidation of sperm sulphydryl groups in mice, leading to male
infertility. Moreover, protein thiol alterations have been shown to result in
perturbation of cell calcium homeostasis and loss of cell viability (Bellomo and
Orrenius, 1985).

It has been reported that membrane sulphydryl groups, when masked
by metal ions like cobalt and copper (in nanomolar concentrations), are known
to inhibit lipoperoxidation and superoxide dismutase activity. This, however,
results in reduced membrane fluidity thereby disturbing the normal sperm
functions (Nivsarkar et al., 1998). It may be noted that although TBARS
production upon antioxidant supplementation was lowered but the GSH level
in the drug-treated series was maintained such that it remained non-
significantly different from the untreated-unsupplemented spermatozoal
samples. Markey et al. (1998) showed that incubation of the initial segment of
rat epididymis in high-oxidative stress conditions caused a 56% decline in GSH
content, a concomitant 240% increase in GSSG concentration, and a 25%
decline in adenosine triphosphate concentration.

Our results showed that GSH level was remained unchanged or slightly
increased but the level of GSSG was shooted upto a higher extent under drug
treatment. Induction of oxidative stress in human sperm suspension using
xanthine-xanthine oxidase system led to an increase in the level of GSSG
following an imposed inhibition on GPx activity (Alvarez and Storey, 1989).
When the spermatozoal suspensions were incubated with nicotine (0.5 & 1
mM) and/or caffeine (5, 7 & 9 mM) the glutathione redox ratio (GSH/GSSG) fell by 49.18 and 60.30% in nicotine treatments and by 20.70, 31.40 and 37.90% in case of caffeine treatments. It was observed that under such a stress, the level of GSSG was raised considerably as it was in 1 mM nicotine concentration, by about 122% (p<0.001) and in 9 mM concentration of caffeine by about 67.40% (p<0.01). From the previous discussion about lipoperoxidation process and with taking the elevated levels of GSSG and decreased redox ratio into consideration, it may lead to believe that nicotine and caffeine are potent and mild peroxidative agents to sperm membranes, respectively.

In different biological systems, the GSH redox status (GSH/GSSG) plays an important role in protecting the cells against oxidative damage (Inoue et al., 1989). A high GSH/GSSG ratio will help spermatozoa to combat oxidative insult (Irvine, 1996). The measurement of GSH/GSSG balance is a potential method to evaluate the oxidative stress status in sperm cells. The reducing equivalents necessary for regeneration of GSH from GSSG by GSH reductase (GRD) are provided by NADPH formed by pentose phosphate cycle (Griveau et al., 1995; Storey and Alvarez, 1998).

In human erythrocytes, Nemeth et al. (2001) showed that oxidative stress caused a GSH depletion with an increase in the GSSG which resulted in an elevated GSSG/GSH (or a decreased GSH/GSSG) ratio as compared to the controls. These authors revealed that GSH stability decreased after an in vitro oxidative challenge which suggested a reduced GSH recycling capacity resulting from an insufficient NADPH supply.

It appears that under the severe nicotine and caffeine-induced oxidative stress, the cellular defense mechanisms are activated and thiols (GSH in particular) are added to the intracellular pool from all the intracellular compartments thereby raising non-significantly the total thiol content of the assay mixture. As redox ratio in drug-treated groups was markedly decreased, which is equal to the utilization of GSH molecules and formation of GSSG units, and simultaneously the amount of GSH remained
almost unchanged, indicating therefore, the possibility of more GSH releasing from intracellular compartment. This release stands for restoring the GSH content and is vital for the cells. On the other hand, it was determined that the activities of some GSH-depleting enzymes like glutathione S-transferase, \(\gamma\)-glutamyl transeptidase (as a GSH-oxidase) and GPx were found to be increased. These enzymes are responsible for utilization of GSH molecules when oxidative stress is started and peroxidation products are produced.

GSH has been reported to be synthesized primarily, if not exclusively, in cytoplasmic compartments in the cells, yet, it is utilized for a variety of detoxification, protection, and physiological functions in other compartments, including the nucleus, mitochondrial matrix, endoplasmic reticulum, and extracellular spaces (Meister, 1991). The availability of GSH in these compartments is determined by the complex interaction of utilization, transport, synthesis and reduction of glutathione disulfide (GSSG) and GS-SX mixed disulfides. GSH is supplied to the mitochondria through an energy-dependent transport system that appears to couple GSH uptake to the efflux of metabolic anions. In contrast, supply to the nucleus appears to involve passive diffusion, yet the nuclear pool also appears to remain kinetically distinct in its differential sensitivity to depletion by chemotherapeutic agents. The supply of GSH to extracellular compartments is dependent on efflux of GSH and is also dependent on redox control mechanisms that allow extracellular reduction of GSSG (Smith et al., 1996).

The intracellular compartmentation of GSH has many important implications for cells that are exposed to toxic compounds or to other stresses. The mitochondrial GSH pool of renal proximal tubular cells, for example, is known to comprise approximately 30% of total cellular GSH and has a GSH concentration of approximately 5mM, which is similar to that in the cytosol (Schnellmann et al., 1988). Numerous mitochondrial proteins, including dehydrogenases, ATPases and transport proteins, contain essential sulfhydryl groups that must be in the reduced form to be biologically active. Hence, alterations in the mitochondrial redox status, such as induction of an oxidative
stress by incubation of mitochondria with an endogenous peroxide or with a
drug that generates reduced oxygen metabolites within the mitochondria,
produces oxidation of GSH and loss of function (Smith et al., 1996).

The redox state of a cell may be expressed as the ratio of the
concentration of oxidizing equivalents to the concentration of reducing
equivalents (Muller et al., 1996). The intracellular antioxidant systems form a
powerful reducing buffer which affects the ability of the cell to counteract the
action of pro-oxidant forces. The level of the pro-oxidant molecules and
antioxidant fluxes thus govern the fine redox balance within a cell. Therefore,
an appreciation of the different sources of oxidants and the counteracting
antioxidants (or reducing) system is necessary to understand what factors are
involved in achieving a particular intracellular redox state (Gabbita et al.,
2000).

Glutathione S-transferase (GST) (EC 2.5.1.18) is a 45 KD protein which
is thought to play a paramount physiological role in initiating the
detoxification of potential alkylating agents (Wood, 1970), including
pharmacologically active compounds. It catalyzes the formation of thioether
conjugates from GSH and a broad range of electrophilic compounds and
thereby converts them to more hydrophilic products and enhances their
excretions (Jakoby and Habig, 1980). GST catalyzes the conjugation reactions
in part, by stabilizing the thiolate anion GS− at the active site (Graminski et
al., 1989). The catalytic advantage is clear, as GS− is a superior nucleophile as
compared to the GSH. However, conjugation with GSH is not the only fate
observed for electrophiles, that are recognized by GST (Siamak et al., 1999).

Gopalakrishnan et al. (1998a) established that both Pi and Mu classes
of GSTs are present on goat spermatozoa and differ in their pattern of
distribution on the cell during epididymal maturation and capacitation. It was
also demonstrated that the rate of acrosome reaction is reduced in the
presence of anti- PiN and anti-MuN antisera. This finding indicates the direct
involvement of GSTs in regulating the secretory events of acrosome reaction.
Glutathione-dependent reduction of hydroperoxides by glutathione peroxidase, a selenoprotein, is believed to be a major defense mechanism of aerobic organisms against ROS attack, which not only catalyze the reduction of ROS like $\text{H}_2\text{O}_2$ but also of lipid hydroperoxides (Awasthi et al., 1975). Zhao et al. (1999) later established that GSH-dependent reduction of hydroperoxides was not limited to the se-dependent GPxs. Evidence is presented for the existence of multiple isoforms of GST, that possess both transferase and se-independent peroxidase activity (Rao and Shaha, 2000).

During the present experimentation, CDNB was used as an electrophile to measure the GST activity in the variously treated spermatozoal samples. GST catalyzes the reaction of CDNB with thiol group of GSH thereby neutralizing its electrophilic sites and rendering a product that is more water soluble (Habig et al., 1974). Our data revealed that the activity of GST was found to be elevated significantly in both nicotine and caffeine-treated series. As these two agents could induce lipoperoxidation in various media, such increase in GST activity may result as a defense response to the released peroxidative products in the assay mixture.

It has been demonstrated by others that the increase in oxidative stress due to reactive oxygen radicals can induce the GST activity (Hayes and Pulford, 1995; cited in Gopalakrishnan and Shaha, 1998). The increased GST activity in the present work also indicates the effective participation of GST or GSH-dependent defense system against oxidative stress induced by compounds like nicotine and caffeine. It can be speculated that GST, beside its role in removing ROS from the samples, could conjugate nicotine and/or caffeine molecules to GSH and thus ultimately get those stabilized and/or excreted in less toxic form; leaving the cell free from their deleterious effects. The GST activity of germ cells has been shown to be increased following exposure to $\text{H}_2\text{O}_2$, which is used as a promoter of peroxidation process. If this increase in activity is inhibited with suitable inhibitors, the formation of LPO products is augmented, resulting in germ cell apoptosis (Rao and Shaha, 2000).
Compounds formed endogenously as well as the exogenous substances introduced into the cells may form GSH conjugates (GSx), e.g. leukotriene A, an epoxide derived from arachidonic acid. GSx are transported across cell membranes, and are substrates of γ-glutamyl transpeptidase (GGT), which converts them to S-substituted cysteinylglycine (Meister, 1983). The schematic metabolic pathways in the glutathione metabolism and its recycling and the role of GST are mentioned in Fig. A.

Fig. A: Outline of GSH metabolism. The cellular turnover of GSH involves its intracellular synthesis from glutamate, cysteine and glycine catalyzed by γ-glutamylcysteine synthetase (1) and GSH synthetase (2), followed by transport of GSH (3) and its conversion by membrane bound γ-glutamyl transpeptidase (4) to cysteinylglycine (CysH-Gly) and γ-glutamyl amino acids. Cleavage of cysteinylglycine to cysteine (CysH) and glycine (gly) may be catalyzed by membrane-bound dipeptidase (followed by transport of the free amino acids) or may occur intracellularly after transport of the dipeptide (5). Transformed γ-glutamyl amino acids (6) are converted by γ-glutamyl cyclotransferase (7) to amino acids and 5-oxoproline; the latter in decyclized by 5-oxoprolinase (8) to glutamate. Glutathione reacts intracellularly with a variety of compounds of exogenous and endogenous origin (X) in reactions catalyzed by GSH transferases (9) to form GSH S-conjugates. These are transported (10) and follow pathway similar to those involved in GSH turnover (4,6, and 11), S-substituted derivatives of cysteine are acetylated (12) to form mercapturic acids, which are transported out of cells (13). Intracellular GSH is converted to GSSG in transhydrogenation reactions (14), in reactions catalyzed by GSH peroxidases (15), and by reaction with free radicals (16). GSSG is converted to GSH by glutathione reductase (17) (Adopted from Meister, 1983).
Suppression of sperm GST activity leads to lipoperoxidation-induced membrane damage, as reflected in the loss of motility, inhibition of acrosome reaction and reduction of the ability of the inhibitor-treated sperm to fertilize oocytes in vitro (Gopalakrishnan and Shaha, 1998). These authors suggested that sperm GST forms an important part of the defense machinery of spermatozoa and is arguably at a more advantageous location on the sperm head in comparison to other known defensive systems like GPx and SOD situated in the sperm mid-piece. In an attempt by Aniya and Naito (1993), it was substantiated that microsomal GSH S-transferase is activated by ischemia/reperfusion-induced oxidative stress in rat liver, and resulted in disulfide bond formation. On the contrary, the data obtained by other investigators indicate that the activity of GST was inhibited after induction of lipoperoxidation (Harris and Stone, 1988; Hassan et al., 1995). The inhibition was modulated by supplementation of antioxidants. However, Tampo and Yonaha (1990) reported that vitamin E and glutathione are required for preservation of microsomal GST from oxidative stress.

In the present work, the kinetic analyses further revealed that addition of nicotine showed a non-competitive mode of inhibition of GST activity. The substrate affinity constant (k_m) of the reaction remained unchanged throughout and was calculated to be 31.746 mM. The V_max of the reaction was considerably elevated in nicotine treatment. The antioxidant supplementation resulted in a lowered GST activity. The most effective antioxidant was considered to be trolox when added (singly). On the other hand, caffeine additions demonstrated a competitive mode of action on GST function. The V_max of this reaction was remained unchanged and found to be 1.091 μmoles CDNB-GSH conjugates/mg prot/min. Moreover, caffeine incubations caused a reduction in the value of k_m in a dose-dependent manner. Supplementation of antioxidants to such samples resulted in a marked increase in the k_m values. Amongst these, trolox again was considered more effective than other antioxidants. It may be speculated that the antioxidants could scavenge and
eliminate the ROS and/or lipoperoxidation products, and therefore due to lack of sufficient target substances for GST, its activity was reduced markedly. The lowered activity of GST indicates the less oxidative stress in spermatozoal samples.

Glutathione reductase, GRD (NADPH: oxidized glutathione oxidoreductase, EC 1.6.4.2) is known to catalyze the reduction of GSSG to GSH, that needs NADPH as a reducing co-factor. The main metabolic pathway leading to the production of NADPH in cells is the pentose phosphate cycle in which the enzyme glucose-6-phosphate dehydrogenase (G6PDH) transforms glucose-6-phosphate into the phospho-6-gluconolactone with the production of NADPH (Griveau et al., 1995; Storey and Alvarez, 1998).

\[
\text{GSSG + NADPH + H}^+ \xrightarrow{\text{GRD}} 2 \text{GSH} + \text{NADP}^+ 
\]

GSH, in turn, can reduce a wide variety of disulfides by transhydrogenation (Ziegler, 1985). Li (1975) reported that the spermatozoa which contained glutathione also exhibited substantial glutathione reductase and glutathione peroxidase activities. The presence of such components in the sperm cells indicate the potential importance of glutathione cycle in human spermatozoa. The present work reports that the GRD activity under the nicotine-induced oxidative stress was significantly depressed but this activity in caffeine treatments, however, was remained almost unchanged. On the other hand, the activity of G6PDH as a key enzyme in formation of NADPH was found to be decreased in nicotine treatment and, on the contrary, increased in caffeine treatment (Figs. 136 & 137).

G6PDH is known to be very susceptible to oxidative damage (Oliver et al., 1987; Griveau et al., 1995), and its inactivation may lead to the blockade of pentose phosphate pathway, and a resultant decrease in NADPH activity in the cell leads to a decrease in the antioxidative protection exerted by the glutathione-dependent defense machinery. This has been reported in other biological models and also in physiological processes like ageing (Noy et al.,
1985; Starke-Reed and Oliver, 1989). The inhibition of G6PDH may be one factor leading to a decrease in NADPH content of human sperm cells (Griveau et al., 1995).

In rats fed a high-fat diet following nicotine administration, it was seen that the levels of lipid hydroperoxides were elevated and the activity of scavenging enzymes like GRD, SOD, and CAT was found to be decreased under the oxidative stress (Ashakumary and Vijaymmal, 1996). Interestingly, Ferrandi et al. (1990) showed that in mouse spermatozoa, the enhanced GRD and GPx activities induced by lipoperoxidation increased the NADPH production from the pentose phosphate shunt pathway, while in rabbit spermatozoa the GRD activity and NADPH productions were much lower. The activity of GRD was reported to be unchanged under oxidative stress, whereas the activity of G6PDH was inhibited significantly, resulting in a lowered formation of NADPH in human sperm samples (Griveau et al., 1995).

From the above findings, it may be speculated that the sensitivity and efficacy of glutathione reductase by which it can convert GSSG to GSH molecules and also the rate of NADPH, depending on G6PDH activity, varies from species to species in mammalian samples and also may be different relative to the source of oxidative attack and its extent. In case of caffeine, although the activity of G6PDH increased significantly but the GRD activity was remained almost unchanged. In this connection, it may be suggested that caffeine could induce a mild oxidative stress in the medium and according to that a mild inhibition was also imposed on GRD activity and that even in the presence of sufficient amount of co-factor NADPH, it still could not increase its activity.

The antioxidant supplementation could increase the GRD activity in nicotine (0.5 & 1 mM) treated samples. The most prominent effect was seen in combination of ascorbate and trolox (p<0.01) as compared to the respective drug-treated spermatozoal samples. In the case of caffeine, the combined effect caused an elevation only in the 5 and 7 mM concentration as compared
to the control data. It appears that the complex between lower concentrations of caffeine and combined concentration of ascorbate and trolox resulted in a better result to induce the GRD activity to form extra GSH molecules via GSH recycling.

Cysteinylglycine formed in the transpeptidase reaction of glutathione is split by dipeptidase (EC 3.4.3.5) into cysteine and glycine. The dipeptides are thought to be transported and hydrolysed intracellularly (Meister and Anderson, 1983). It has been shown that there is a substantial cytosolic dipeptidase activity (Das and Radhakrishnan, 1973; cited in Meister and Anderson, 1983). In order to monitor the effect of nicotine and caffeine on the activity of the enzyme dipeptidase, the amount of glycine liberated as one of the products of the enzymatic reaction was quantitated chromatographically. It was registered that nicotine depressed the activity of dipeptidase but non-significantly (p>0.05), while caffeine treatments (5,7 & 9mM) caused a statistically significant increase in the dipeptidase activity (p>0.05, p<0.05 & p<0.05, respectively). Any change in the activity of membrane-bound dipeptidase may be translated into an alteration in the rate of glutathione production within γ-glutamyl cycle. Antioxidant additions could not register any alteration in the activity of dipeptidase in control and nicotine-treated spermatozoal samples. Supplementation of antioxidants, singly and/or in combination form in caffeine-treated samples resulted in an increased activity of dipeptidase when compared to control data. The dipeptidase was not considered as a sensitive enzyme to oxidative stress as the ROS scavenging activity of the antioxidants used was not much effective in nicotine-treated samples which were responsible for inducing a severe lipoperoxidation in the medium.

Biosynthesis of glutathione occurs in virtually all living cells. The first step in the synthesis is catalyzed by gamma-glutamylcysteine synthetase (GGCS) (EC 6.3.2.2) from glutathione, cysteine and glycine (Yip and Rudolph, 1976; Meister, 1985). The enzyme utilizes L-cysteine and L-α-amino butyrate
equally well. In the present investigation, L-α-amino butyrate was used as the substrate of the reaction so as to avoid complications associated with spontaneous oxidation with L-cysteine. This enzymatic reaction utilizes an ATP molecule, thereby liberating an inorganic phosphorus which was estimated during the present experiment. Nicotine incubations (0.5 & 1 mM) resulted in a significant (p<0.01) reduction in the GGCS activity. On the other hand, caffeine additions caused no significant alteration. The GGCS is known to be inhibited non-allosterically by glutathione under conditions similar to those that prevail *in vivo* thus indicating a physiologically significant feedback mechanism (Richman and Meister, 1975). Glutathione also inhibits the GGCS activities of fetal liver and Novikoff hepatoma (Wirth and Thorgeirsson, 1978). Decreased GGCS activity occurs in the erythrocytes of certain glutathione-deficient sheep (Young and Tucker, 1983).

It was demonstrated that antioxidant supplementation could improve the activity of inhibited GGCS under nicotine treatment which yielded a severe lipoperoxidation in the assay mixture. It seems that nicotine had a direct inhibition on GGCS activity which led to a block in GSH biosynthesis that could result in cellular damage. Antioxidants proved to play a corrective role in reactivating the enzyme activity. In the case of caffeine, only in 9 mM concentration a remarkable elevation was observed in the GGCS activity after addition of antioxidants which was more even than control value. This effect may be because of a synergistic effect between caffeine molecules and antioxidants in the assay mixture by which more GGCS activity was obtained.

Gamma-glutamyl transpeptidase (GGT) (EC 2.3.2.2) is a key enzyme of gamma-glutamyl cycle (Meister, 1985; Sastry, 1991). It reversibly breaks down glutathione. It can catalyse three type of reactions: (a) transpeptidation, in which gamma-glutamyl moiety is transferred to an acceptor; (b) auto transpeptidation, in which gamma-glutamyl moiety is transferred to glutathione to form gamma-glutamyl glutathione; and (c) hydrolysis, in which the gamma-glutamyl moiety is transferred to water. The GSH, S-substituted glutathione and other gamma-glutamyl compounds are the substrates while
the L-isomer of cysteine, methionine, and dipeptides and especially the amino acylglycine, act as good acceptors (Meister and Anderson, 1983).

Data obtained from the work by Funahashi et al. (1999) revealed that glutathione content in rat eggs decreased between the process of sperm nucleus decondensation and pronuclear formation, probably due to the increased activity of gamma-glutamyl transpeptidase. Our results show that nicotine treatment caused a very significant elevation in the GGT activity (p<0.001), but caffeine could not alter the GGT activity significantly in spermatozoal samples.

Markey et al. (1998) reported that in a high-oxidative stress condition, the expression of GGT mRNA was increased depending on the concentration of oxidizing agents and the type of ROS generated. This elevated activity of GGT was responsible for oxidizing the GSH molecules to GSSG units. The apparent glutathione oxidase activity of GGT was reported earlier by Griffith and Tate (1980), in which it is indicated that GGT initiates the oxidation of GSH by the mechanism shown in Fig. B.

1. \( \text{GSH} \xrightarrow{\text{GGT}} \text{GLU} + \text{CYSH} \xrightarrow{} \text{GLY} \)

2. \( \text{CYSH} \xrightarrow{} \text{GLY} + \text{GSH} \xrightarrow{} \text{CYS} \xrightarrow{} \text{GLY} \)

3. 2 \( \text{CYSH} \xrightarrow{} \text{GLY} \xrightarrow{} \text{CYS} \xrightarrow{} \text{GLY} \)

4. \( \text{CYS} \xrightarrow{} \text{GLY} + \text{GSH} \xrightarrow{} \text{GSSG} + \text{CYSH} \xrightarrow{} \text{GLY} \)

5. \( \text{CYS} \xrightarrow{} \text{GLY} + \text{GSH} \xrightarrow{} \text{CYS} \xrightarrow{} \text{GLY} + \text{CYSH} \xrightarrow{} \text{GLY} \)

Fig. B: Shows mechanism of disulfide formation from GSH in presence of GGT. The only step requiring the enzyme is step (1), which forms the true oxidation catalyst, cysteinlyglycine. In step (2), GSH would yield \( \gamma \)-glutamyl GSH. Step 4 yields the ultimate product, GSSG, non-enzymatically. The reaction 5 is required, if only the step (3) is responsible for oxidation.
It can be speculated that the elevated activity of GGT is partially responsible for increased amount of GSSG unit in nicotine-treated spermatozoal samples. It has also been documented that GGT activity can give rise to redox reactions, leading to the production of ROS and lipoperoxidation (Stark et al., 1993; Drozdz et al., 1998; Maellaro et al., 2000). Further, Perego et al. (1997) substantiated that GGT-dependent pro-oxidant reactions are involved in inhibition of proliferation in ovarian cancer cells. Significant levels of GGT have been reported in a number of human malignant neoplasms, e.g. colon cancer (Murata et al., 1997).

Expression of GGT activity can provide cancerous cells with an additional source of hydrogen peroxide, and that such pro-oxidant reactions are capable to modify related protein thiols at the cell surface level, leading to a depressed proliferation status of melanoma cells (Maellaro et al., 2000). It may be inferred from the above findings and our results that nicotine as a potent oxidant could increase the GGT activity, resulting in generation of ROS and subsequent lipoperoxidation in human spermatozoal samples.

The elevated GGT activity upon nicotine treatment was found to be depressed following addition of antioxidants. It may be postulated that antioxidants with their capacity to absorb the nicotine-derived ROS in the medium could depress the augmented activity of GGT. The elevated GGT activity may be translated into a more GSH-oxidase activity in the assay mixture. In this connection, it was shown that addition of trolox was more effective than other antioxidants. Caffeine also could increase the activity of GGT but not to any remarkable extent, and still antioxidant effects were seen to be of great importance to reserve the GSH molecules in the spermatozoal samples.

Human spermatozoa possess enzymatic defense systems such as glutathione peroxidase, catalase and superoxide dismutase to counteract the toxic effects induced by oxygen free radicals (Alvarez and Storey, 1989; Griveau et al., 1995). Glutathione peroxidase (GPx) (EC 1.11.1.9) is a key
enzyme in glutathione metabolism. Hydrogen peroxide, the by-product of SOD action, is eliminated either by catalase or by GPx. The latter enzyme that also is referred as GPx1 has a powerful antioxidant action. Lipid $H_2O_2$ and hydroperoxides are reduced via GPx since catalase is effective only against $H_2O_2$ molecules. This suggest that GPx has a pivotal role in the cellular antioxidant protection (Guerin et al., 2001).

Genetic inactivation of GPx1 resulted in growth retardation, presumably due in part to reduced mitochondrial energy production as a product of increased oxidative stress (Esposito et al., 2000). Alvarez and Storey (1989) had reported that GPx plays a role in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipoperoxidation. They demonstrated that inactivation of GPx allows lipid hydroperoxides to accumulate and therefore increases the peroxidation rate in sperm membrane.

Our results clearly revealed that lower concentrations of nicotine (0.5 mM) and caffeine (5 & 7 mM) were activated the GPx activity. This effect was more pronounced in nicotine than caffeine treatment. As GPx is responsible for neutralizing and removing $H_2O_2$ and lipid hydroperoxides from the medium, therefore, any elevation in the activity of GPx can be translated into the presence of oxidative stress and subsequently lipoperoxidation in the treated samples. Ferrandi et al. (1990) reported an enhanced glutathione peroxidase activity, induced by spontaneous lipid peroxidation in mouse sperm cells during in vitro incubation. It has been shown that the GPx activity and glutathione content were increased in rat following increase in the concentration of lipid hydroperoxides in medium (Ashakumary and Vijayammal, 1996). We have reported that, the GPx activity after addition of nicotine at a higher concentration (1mM) and also caffeine (9 mM) was found to be depressed, which was even less than the control value. Under these conditions the observed reduction in the activity of GPx may be linked to a direct inhibition of glutathione peroxidase by ROS formed or LPO by-products. Such an inhibition by hydroperoxides has been described in the
mammalian cells and shown to be associated to an oxidative modification of the enzyme protein (Ochi, 1990). The selenium atoms present at the catalytic site of the GPx undergo a redox cycle during the enzymatic detoxification of lipid hydroperoxides, between a reduced (selenol) and an oxidized form (selenic acid) (Flohe, 1982; cited in Ochi, 1990). It can be hypothesized that the increase of the oxidation level/ROS formation of the cell slows down the return to the reduced state of selenium, leading to a reversible inhibition of the enzyme whereas antioxidant supplementation could improve the change in the GPx activity. Another possibility is the alteration of the peptide chain in enzyme structure by hydroxyl radicals and \( \cdot \text{O}_2 \) which may induce fragmentation of the GPx (Blum and Fridovich, 1985; Cited in Gaullier et al., 1994).

The result presented in this study is in agreement with those reported by Griveau et al. (1995) in which treatment with hydrogen peroxide as a potent ROS was shown to inactivate several enzymatic activities involved in the antioxidant defense system including GPx, SOD and G6PDH in human sperm suspensions.

GPx activity was increased under 0.5 mM nicotine concentration-induced oxidative stress but upon antioxidant supplementation it was seen to be depressed due to elimination of formed ROS by the absorbing capacity of antioxidants in the assay mixture. On the other hand, due to a severe oxidative stress after 1 mM nicotine addition, an inhibition of GPx activity was observed that was modulated by the antioxidants. In case of caffeine (5 & 7 mM), the condition seemed to fulfil that of the 0.5 mM nicotine concentration. 9 mM concentration of caffeine proved to be as a mild oxidant which could diminish the GPx activity, but under antioxidant functions, this inhibitory effect was blocked. The combination of ascorbate and trolox was found to act efficiently in all the spermatozoal samples.
Adenosine Triphosphatases (ATPases)

The present work is aimed to evaluate the functional status of the human sperm cells. For this, the activities of total ATPase, Na⁺-K⁺-ATPase, Ca²⁺- and Mg²⁺- dependent ATPases were monitored in the presence of nicotine, caffeine and antioxidants (1 mM ascorbate, 5 mM glutathione and 0.02 mM trolox). The loss of sperm motility and the capacity for sperm-oocyte fusion have been associated with reduction in the membrane fluidity and increase in lipid peroxidation which, in turn, may lead to the inhibition of certain membrane-bound enzymes, especially the adenosine triphosphatases (Sharma and Agarwal, 1996). Lipid peroxidation also impairs the cell membrane ion exchange that is essential for maintaining the normal sperm movements (Rao et al., 1989).

The ATPase : Na⁺-K⁺-ATPase, Ca²⁺-ATPase and Mg²⁺-ATPase are involved in regulating the calcium homeostasis in various cells as well as in mammalian sperm cells (Meizel, 1984; Sidhu and Guraya, 1989a; Roldan and Fleming, 1989; DasGupta et al., 1994; Woo et al., 2000). Any interference in the production of ATP and also the decline in ATPase activity could inhibit the motility of spermatozoa (Kalla and Vasudev, 1981). It may be assumed that sperm plasma membrane is ‘biologically forzen’ when spermatozoa leave the male’s body and its ‘defrosting’ process represents capacitation (Yanagimachi, 1994). Calcium is believed to be the molecule that triggers capacitation which required the ATPase activities in both plasma and organelar membranes.

The activity of total ATPase in the sperm cells was measured and found to be lowered by the effect of nicotine (1mM) by about 18.76% (p<0.05). On the other hand, caffeine exerted an augmentative effect on the total ATPase activity in spermatozoal membrane fractions (22.10%, p<0.05). Antioxidant supplementation played an improving role in total ATPase activity in the drug-treated samples leading to a proper and regulated function in the
membranes. The combination of trolox and glutathione showed a better result in maintaining the ATPases in the cell membranes.

The Na\(^+\)-K\(^+\)-ATPase, localized in the sperm plasmalemma is known to be activated by Mg\(^2+\) and inhibited by Ouabain (Ashraf et al., 1982). It undergoes a cycle of conformational changes as it hydrolyzes the ATP and translocates Na\(^+\) (outwardly) and K\(^+\) (inwardly), thereby balancing the function of Na\(^+\)/K\(^+\) pump (Stryer, 1995; Darnell et al., 1995). Na\(^+\)-K\(^+\)-ATPase is an enzyme, satisfying several criteria for receptor binding, and it perhaps facilitates the contact between spermatozoa and extraneous agents such as certain drugs (Mann and Mann, 1981). The inhibition of Na\(^+\)-K\(^+\)-ATPase resulted in an accumulation of Na\(^+\) in the cell that can be exchanged with extracellular Ca\(^{2+}\) as Ca\(^{2+}\)/Na\(^+\)-antiporter which has been demonstrated in the sperm cells (Rufo et al., 1984; Hang et al., 1990). The calcium ions are necessary for sperm motility and acrosome reaction while sperm cells are in the female's genital tract. Membrane components essential for cell survival e.g. Na\(^+\)-K\(^+\)-ATPase must be in the sperm plasma membrane all the time, but those that will later perform sperm specific functions (e.g. those essential for sperm-oocyte communications) may be added to the sperm plasma membrane (or altered to active form) while spermatozoa are maturing in the epididymis (Yanagimachi, 1994). Our data show that nicotine could decrease the Na\(^+\)-K\(^+\)-ATPase activity non-significantly. This depression may be attributed to the nicotine-induced oxidative stress in spermatozoal membrane fraction, which led to inactivation of ion pump in the spermatozoal membranes. Kako et al. (1988) had found that the activity of Na\(^+\)-K\(^+\)-ATPase was reduced proportionally to the concentration of hydrogen peroxide and the duration of incubation. There was a decrease in –SH content and turnover rate of enzyme preparation, while MDA and conjugated dienes were generated from the membrane lipids in the course of incubation. From the above findings, it may be suggested that a potential oxidant like nicotine can alter the Na\(^+\)-K\(^+\)-ATPase activity over a long exposure period and mask the –SH groups of
membrane leading to malfunction and/or inactivators. Other investigations demonstrated that the activity of Na\(^+\)-K\(^+\)-ATPase is depressed under oxidative stress (Yesilkaya and Yegin, 1998; Kurella et al., 1999; Jain and Lim, 2000). The Na\(^+\)-K\(^+\)-ATPase activity was inhibited significantly under high glucose concentration-induced lipid peroxidation in RBC from diabetic patients (Jain and Lim, 2000). Any changes in the cholesterol/phospholipid ratio would result in an inactivated Na\(^+\)-K\(^+\)-ATPase (Kanbak et al., 2001).

Recently, a new isoform of Na\(^+\)-K\(^+\)-ATPase (alpha 4 isoform) has been identified in the mammalian spermatozoa. The inhibition of this isoform alone can eliminate the sperm motility (Woo et al., 2000). This biological function reveals a critical role for Na\(^+\)-K\(^+\)-ATPase in sperm movement and activation which depends on different conditions. Moreover, it has been shown that antioxidant supplementation could improve the Na\(^+\)-K\(^+\)-ATPase activity. It may be speculated that antioxidants could absorb the nicotine-induced free radical formation in the samples resulting in protection of enzyme sulfhydryl groups and proper function of ion pump. This effect is exactly against the ability of lipoperoxidation in altering the ion exchanges across the plasma membranes. Antioxidant supplementation including ascorbate and \(\alpha\)-tocopherol could inhibit the decrease in the activity of Na\(^+\)-K\(^+\)-ATPase due to ultraviolet (UV)-B exposure-induced lipoperoxidation in the rat lens (Reddy and Bhat, 1999). UV-B irradiation resulted in a decrease by about 57% in this ATPase. Oxidative modification of Na\(^+\)-K\(^+\)-ATPase was eliminated following antioxidant supplementation in mammalian brain, kidney and erythrocytes (Yesilkaya and Yegin, 1998; Kurella et al., 1999; Jain and Lim, 2000).

On the other hand, caffeine showed a slight increase (\(p>0.05\)) in the activity of Na\(^+\)-K\(^+\)-ATPase in spermatozoal membrane fractions. Karacagil et al. (1990) established that addition of caffeine to the poor sperm samples such as samples from oligozoospermic men caused stimulation in the activity of Na\(^+\)-K\(^+\)-ATPase by 13.78%. They had reported that the optimal concentration of added caffeine would appear to be about 7.2 mM, while in samples treated
with either lower or higher caffeine concentrations, no significant differences in sperm motility was apparent after 10 or 30 days of frozen storage (Barkay et al., 1977, 1984). It has been reported that, caffeine failed to stimulate the Na⁺-K⁺-ATPase activity in normozoospermic men (Karacagil et al., 1990). They have suggested that the concentration of caffeine in the seminal plasma of normal men would be enough to stimulate the ATPase activities of sperm cells. Although caffeine is known as a potent phosphodiesterase inhibitor and increases the intracellular level of cAMP inducing stimulation of sperm metabolism and sperm motility (Amelar et al., 1980; Fayed, 1996; Lopez and Alvarino, 2000), but some of its pharmacological activities can not easily be explained on this basis. A competitive manner of effect was registered in nicotine and caffeine-treated series in substrate kinetic studies. The V_max was remained unchanged and calculated to be 5.820 μM Pi.mg prot.⁻¹.min⁻¹. The k_m values were reduced upon caffeine additions. On the other hand, an increase in k_m values was observed with nicotine incubations. The antioxidant supplementation caused further decrease of k_m in caffeine-treated samples and also decrease of k_m in nicotine-treated samples. The unchanged V_max and lessening the values of k_m in these systems show a better function in drug-treated spermatozoal membrane fractions upon antioxidant supplementation. It was found that trolox played a better role in activation of Na⁺-K⁺-ATPase activity. A synergistic effect may be suggested between the antioxidants and caffeine in stimulating the ATPase system.

Ca²⁺-ATPase located in the plasma membrane helps to maintain low intracellular concentration of Ca²⁺ by either pumping this ion out of the cell (Penniston, 1983; Schatzmann, 1985) or into an organelle, such as sarcoplasmic reticulum (Shamoo, 1985) and/or acrosome sac (Gordon et al., 1978). Many investigations demonstrated the presence of an outward-directed Ca²⁺-pump (Ca²⁺-ATPase) in the head and flagellar membranes of mammalian spermatozoa (Peterson et al., 1983; Garcia et al., 1991). The inhibition of this enzyme would facilitate the Ca²⁺ accumulation in sperm cell which is required
for acrosome reaction (DasGupta et al., 1994; Li and Chen, 1996). Increasing age caused a significant decrease in Ca\textsuperscript{2+}-ATPase activity resulting in Ca\textsuperscript{2+} accumulation in brain tissue (Hanahisa and Yamaguchi, 2001). Bradley and Forrester (1985) postulated that Ca\textsuperscript{2+}-ATPase might only be responsible for the fine tuning of Ca\textsuperscript{2+} level while other mechanisms for example, antiporters, being the major regulators of Ca\textsuperscript{2+} fluxes. Roldon and Fleming (1989) pointed out the possible involvement of Ca\textsuperscript{2+}-ATPase in the regulation of intracellular Ca\textsuperscript{2+} during capacitation and subsequent acrosome reaction of guinea pig spermatozoa using Ca\textsuperscript{2+}-ATPase antagonists. It is well established that a massive influx of Ca\textsuperscript{2+} takes place during the capacitation in sperm cell (Yanagimachi, 1981). Our results indicate that nicotine addition (1mM) could inhibit the Ca\textsuperscript{2+}-ATPase activity significantly (23.79%, p<0.05). As Ca\textsuperscript{2+}-ATPase is a membrane-bound enzyme, so that any alteration in membrane structure by oxidative agents like nicotine could translate into an inactivation for this enzyme. Lipoperoxidation is known to inactivate certain enzymes, both cytosolic and membrane-bound, such as Ca\textsuperscript{2+}-ATPase (Ohata et al., 1989; Poznansky, 1990; Jain and Lim, 2000). A number of aldehydes are formed which can attack the primary amino groups in proteins resulting in an inactivation. Malonyldialdehyde as a binfunctional aldehyde can cross link proteins in biomembranes (Thomas and Poznansky, 1990).

Increased lipoperoxidation depressed the activity of plasma membrane-bound ATPase activity in CCl\textsubscript{4}-treated rat liver significantly (Mourelle and Meza, 1990). In 1985, Scherer and Deamer had reported that peroxidation can directly inhibit the Ca\textsuperscript{2+}-ATPase activity. The decline may be due to oxidation of -SH groups, as suggested by the ability of reducing agents to prevent inhibition and decline in sulphhydryl content of microsomes as subjected to H\textsubscript{2}O\textsubscript{2} and other oxidants, like iron-ascorbate complex. This is the mechanism by which the function of Ca\textsuperscript{2+}-ATPase could be affected by the alteration in the cellular redox potential. Other investigators have also been demonstrated that oxidative inhibition of Ca\textsuperscript{2+}-ATPase in the presence of free radicals was
lipid independent and took place via oxidation of membrane sulfhydryl groups (Bellamo et al., 1983; Okabe et al., 1983: cited in Scherer and Deamer, 1985). Castilho et al. (1996) pointed out that the oxidative damage to the activity of Ca²⁺-ATPase in the sarcoplasmic reticulum membrane is not mediated by lipoperoxidation cascade or thiol oxidation. This impairment of function may be related to amino acid oxidation and fragmentation of the enzyme protein. The work by Kanbak et al. (2001) revealed that whenever a drop is found in the cholesterol/phospholipid ratio in the membrane structure, the Ca²⁺-ATPase activity would be lowered.

Using combination of glutathione and stobadine resulted in an increase in the inhibited Ca²⁺-ATPase activity under oxidative stress induced by free radical generating systems like Fe²⁺-EDTA complex (Lehotsky et al., 1999). In diabetic neuropathy, the use of antioxidant could delay or inhibit the decrease in Ca²⁺-ATPase activity resulting in an improvement in the RBC membrane function (Jain and Lim, 2000). In the present work, antioxidant supplementation could increase the Ca²⁺-ATPase activity and addition of the combination of trolox and GSH approached nearly the control values.

Addition of ascorbate+GSH, ascorbate+trolox and trolox+GSH resulted in more activity of Ca²⁺-ATPase by 17.71, 22.25 & 29.74% (p<0.05), respectively, as compared to the treated samples by nicotine. When caffeine (7mM) was added to spermatozoal samples, an elevation in Ca²⁺-ATPase activity occurred significantly (27.56%, p<0.05). It has been approved that caffeine is a mild oxidant to biomembranes, but in this connection, it can be postulated that low level of oxidized lipids and/or free radical generated under experimental conditions did not markedly affect the Ca²⁺-ATPase activity. It has also been reported that caffeine is able to stimulate the Ca²⁺-ATPase activity of calcium intracellular stores to accumulate the increased cytosolic Ca²⁺ in snail neurons (Collins and Thomas, 2001). The cytosolic Ca²⁺ is reported to be transported actively into the acrosome by the function of a
Ca²⁺-ATPase (ionic pump) and then the accumulated Ca²⁺ could be released from acrosome pool via an IP₃-gated calcium channel (Dragileva et al., 1999).

The alteration of intracellular Ca²⁺ as a result of modulation of cellular calcium transport may be a possible mechanism by which methylxanthines like caffeine enhances the activation and movement of sperm cells (Hong et al., 1985). It may be speculated that on one hand, caffeine additions resulted in a Ca²⁺ influx (may be via ion channels) and on the other caused an increase in the activity of plasma membrane-bound Ca²⁺-ATPase to extrude the additional Ca²⁺ from the cell. Moreover, it may also be speculated that mild caffeine-induced free radical formation participated in the increase of Ca²⁺ intracellularly via inducing the protein tyrosine kinase activity in sperm cells (Aitken et al., 1998; De Lamirande et al., 1998) which can be responsible for the augmented Ca²⁺-ATPase activity. Karacagil et al. (1990) had shown that using caffeine resulted in an increase in the Ca²⁺-ATPase activity in oligoasthenic spermatozoa. In buffalo spermatozoa, the Ca²⁺-ATPase activity is evident in plasma membrane from sperm heads and tails, although the activity is greater in the latter (Sidhu and Guraya, 1993). These authors reported that a calmodulin-like protein isolated from buffalo seminal plasma increased the Ca²⁺-ATPase activity of plasma membrane from sperm heads and tails by 128 and 136%, respectively. It may be speculated from this finding that intracellular calmodulin is also involved in the activation of Ca²⁺-ATPase in caffeine – treated samples. Antioxidant supplementation did not register a significant alteration in Ca²⁺-ATPase activity in caffeine – treated spermatozoal membranes, as compared to the respective drug-treated samples. However, the antioxidant supplementation (singly / in combination) raised the activity of ATPase non-significantly as compared to the control data.

Antioxidants may be considered as helper agents to maintain the proper activity of membrane-bound enzymes. Our substrate kinetic data reveal that nicotine and caffeine showed an uncompetitive manner of action on Ca²⁺-ATPase activity in the various samples. In nicotine – treated samples the
values of $V_{\text{max}}$ and $k_m$ were decreased but upon antioxidant supplementation an inhibition was registered in these suppressions. On the other hand, caffeine treatments registered an augmentation in both $k_m$ and $V_{\text{max}}$ values and this increase was further induced under antioxidant supplementation. The augmented values of $V_{\text{max}}$ and $k_m$ exhibited an increase in ATPase activity and on the contrary, the dropped values of $V_{\text{max}}$ and $k_m$ exerted an inhibitory role for the agent used upon ATPase activity. Antioxidants with their ROS absorbing capacity could improve the ion-exchange process across the sperm plasma membrane, leading to a completely normal function and intracellular homeostasis.

Usui and Yanagimachi (1986) have described some cytochemical studies which localized a Mg$^{2+}$-ATPase on the outer acrosomal membrane and overlying plasma membrane of guinea pig spermatozoa. Meizel (1984) has further described that, decreased Na$^+$-K$^+$-ATPase and Mg$^{2+}$-ATPase (H$^+$-pump) activities are involved in the membrane events of the golden hamster acrosome reaction/capacitation process. Ca$^{2+}$ inhibits the activity of Mg$^{2+}$-ATPase (proton pump) and thus facilitates water influx into acrosome inducing approximation of the opposing membranes and leading to vesiculation and discharging the hydrolytic enzymes required for penetrating the egg layers by sperm cell.

During the present course of investigation, a significant decline (25.59%, $p<0.05$) in the activity of Mg$^{2+}$-ATPase was observed in human sperm cell membrane treated with 1 mM nicotine as compared. A common characteristic of ATPases is that if catalyzes the same overall reaction (the hydrolysis of ATP to adenosine diphosphate and inorganic phosphate) and utilizes the free energy changes of this reaction for the transport of ions against an electrochemical gradient across the membrane in which it is located. Nicotine, as mentioned earlier, is a potent oxidant to biomembranes. Nicotine-induced peroxidation can alter the interactions between lipid fractions and proteins in the cell membrane. Blazovics et al. (1989) had
reported that the presence of lipoperoxidation cascade in the rat brain plasma membranes results in inhibited Mg\textsuperscript{2+}-ATPase activity which is protected by the action of antioxidants. The activity of Mg\textsuperscript{2+}-ATPase has been reported to be depressed under the ascorbate/Cu\textsuperscript{2+}-induced lipid peroxidation in RBC membrane significantly (Chattopadhyay et al., 1992). Our data showed that antioxidant supplementation, particularly in the combined concentrations could exhibit an improvement in the inhibited Mg\textsuperscript{2+}-ATPase activity. It seems clearly that antioxidants could overcome the imposed oxidation on the integrity and function of Mg\textsuperscript{2+}-ATPase in nicotine-treated membranes. Using antioxidants in combination form to prevent inhibition of ATPase systems during oxidative stress is strongly recommended by Lehotsky et al. (1999).

Caffeine as a methylxanthine agent augmented the Mg\textsuperscript{2+}-ATPase activity in the current study by 29.06% (p<0.05). The supplementation of antioxidants in caffeine (7 mM)-treated samples resulted in a further elevation in the Mg\textsuperscript{2+}-ATPase activity which appears to be due to a synergistic effect between caffeine and the antioxidant on the function of proton pump. Karacagil et al. (1990) have pointed out that Mg\textsuperscript{2+}-ATPase activity of oligoasthenic sperm cells was stimulated after addition of caffeine. The ATPase activities have been reached to the maximum level when 10 mM concentration of caffeine was added to the standard incubation medium for human spermatozoa.

The substrate kinetic studies have revealed that nicotine and caffeine effects demonstrated a competitive manner of action on the activity of Mg\textsuperscript{2+}-ATPase in the samples. The \( V_{\text{max}} \) was calculated to be 6.20 \( \mu \text{M Pi.mg prot}^{-1}.\text{min}^{-1} \), and the \( k_m \) values were found to be increased in nicotine-treated samples, whereas in caffeine-treated samples it was seen to be decreased. Antioxidant supplementation caused a sharp drop in \( k_m \) values of drug-treated spermatozoal membranes which led to the proper functioning for the ATPase.
Motility Test

Spermatozoa, unlike other body cells are endowed with two clearly discernible biological properties, namely the capacity to move fast and to fertilize. This is a remarkable combination of attributes, each inherent in a different constituent structure of a sperm cell. Flagellar motion is a major energy-demanding chain of reactions in spermatozoa, and sperm motility in all its aspects assuredly has first claim upon the provision of energy; whilst other events, such as ion exchange across the sperm surface may need and receive rather less amount from the energy reservoir in the sperm mitochondria (Mann and Mann, 1981). The investigators further described that a spermatozoon has to overcome both the stiffness of the flagellum, i.e. its own mechanical inefficiency, and the resistance of the surrounding semiviscous secretions for continuous vigorous movement. In the ejaculate, each spermatozoon must prevail over the resistance arising from crowding, caused by the close proximity of other sperm cells. To meet these conditions and to enable flagellar motion to develop, a spermatozoon must first transform the chemical energy present in the mitochondrial adenine nucleotide pool into the mechanical energy. It has been demonstrated that mitochondrial DNA 4977-bp deletion is associated with diminished motility and fertilizing ability of human sperm cells (Kao et al., 1995).

A significant sperm motility enables the sperm cell to penetrate through the cervical mucus and to come in close proximity with the ovum while travelling in the female genital tract. The vigorous movement also provides greater propelling power to the sperm cell to penetrate through the egg investments thus resulting in fertilization of the ovum (Yanagimachi and Usui, 1974). Motility correlates directly with the fertilization rates of human oocytes in vitro (Bongso et al., 1989) and with the pregnancy rates after artificial insemination (Tucker et al., 1991; Sharma et al., 1996). Poor sperm motility rather than a low sperm count is the main cause of male infertility which is frequently encountered in assisted reproduction (Cai et al., 1989).
Since sperm motility, both quantitatively and qualitatively plays a vital role in deciding the fertilizing status of semen, the present work was aimed to investigate the effect of nicotine and caffeine with antioxidant supplementation on the spermatozoal motility of healthy non-smokers.

During the course of present investigation, a drastic effect was registered on the rate of sperm motility following incubation with different concentrations of nicotine (0.5 & 1 mM). The percent motile sperm cells was decreased in the nicotine-treated samples as the time of incubation with the drug was increased. The fall in the sperm motility rate in 0.5 mM nicotine addition was 11.90% (p<0.05), but in 1 mM concentration it was found to be 21.42% (p<0.01) as compared to the respective control data at 120 minutes of incubation time. The sperm motility rate was started to decrease significantly at 20 min of incubation time in the 1 mM nicotine addition. In contrast, the decreasing rate of sperm motility began at 40 min of incubation time with 0.5 mM nicotine concentration. In complete agreement with our results, Reddy et al. (1995) have substantiated that, nicotine at concentration of > or = 1 mM significantly decreased the sperm motility characteristics after different period of incubations. On the contrary, Crandall et al. (1989) reported that sperm motility parameters were not affected by the addition of nicotine to semen samples incubated with BWW medium. Smokers have significantly poorer sperm density, a lower percentage of viability, a lower percentage of normal sperm morphology, and a decreased percent of motile sperm cells. These parameters are worse in the heavy smokers (Merino et al., 1998).

Nicotine at concentration of 0.1 mM and above in some way could inhibit the acrosomal loss in human spermatozoal samples resulting in a decrease in sperm penetration of zona-free hamster eggs (Pekarsky et al., 1995). We earlier showed that nicotine could inhibit the activity of ATPases in human spermatozoal membrane fractions which leads to an inhibition on the sperm movement and acrosome reaction as a final part of capacitation process. Antioxidant supplementation has been able to reverse the damaging
effects of nicotine treatment. Moreover, Rothschild (1953) found that nicotine caused a sharp reduction in the spermatozoal oxygen uptake, thus acting as a deterrent to motility rate. The impairment of respiratory metabolism following decreased oxygen uptake after nicotine addition and resultant drop in cAMP level could be responsible for diminished sperm motility. The role of ATP dephosphorylation as the primary and immediate source of energy for sperm motility has been universally accepted (Mann and Mann, 1981; Nelson, 1985; Eddy and O’Brien, 1993). Any interference in the production and decline in the activity of ATPases could inhibit the motility rate of spermatozoa (Kalla and Vasudev, 1981). An inverse correlation has been established between the percent motile sperm cells and the quantity of ROS detected in the semen (Iwasaki and Gagnon, 1992). Nicotine as a potent oxidant is able to alter the sperm plasma membrane and related functions like induction of motility. Mouse and human spermatozoa have long been known to be sensitive to the loss of motility induced by oxygen free radicals (Alvarez and Storey, 1989). Inhibition of peroxidases allows to increase the peroxidation rate and loss of motility in human sperm cells (Alvarez and Storey, 1989). In contrast, Baumber et al. (2000) substantiated that the decrease in equine sperm motility associated with oxygen free radicals occurs in the absence of any detectable increase in lipoperoxidation rate. However, in the present work it appears that increasing nicotine-induced oxidative stress proportionately results in loss of spermatozoal motility. Since the flagellar energy providers, like ATPases, require a sulfhydryl group in the reduced form to be in the active state, increased oxidation process leads to a reduction in their activities.

Antioxidant supplementation (singly/in combination) appeared to maintain or improve the spermatozoal motility of untreated or treated samples via overcoming the oxidative stress induced by nicotine additions. It has been demonstrated that the production of ROS is strongly associated with the loss of sperm motility (Aitken et al., 1989). Seminal plasma from infertile
men has a significantly lower antioxidant capacity than that from fertile men (Lewis et al., 1995). Collectively, it can be said that the influence of antioxidants on sperm plasma membrane is mediated by the stabilization of the disturbed membrane fluidity or neutralization of the toxicity of ROS so as to reduce the oxidative damage to the sperm cells.

Donnelly et al. (1999) had reported that ascorbate in the range of 20-600 μM affects sperm motility adversely in both normozoospermic and asthenozoospermic samples. The percentage of progressive motility, velocity of sperm movement, and sperm head oscillations were reduced. All parameters were significantly reduced to a greater extent in asthenozoospermic samples than that in normozoospermic ones. These authors also reported that trolox had a detrimental effect on sperm motility and velocity. When combination of ascorbate and trolox was added to the spermatozoal preparation, it appeared to have a greater detrimental effect on sperm motility than when either is added alone (Donnelly et al., 1999). Moreover, Baker et al. (1996) substantiated that the combination of ascorbate and α-tocopherol does not have a significant protective effect on the damage to the sperm motility caused by activated polymorphonuclear leukocytes in vitro. These findings emphasize the view that the antioxidant therapy may indeed be a double-edged sword, with negative effects if a certain threshold concentration is exceeded (Tarin et al., 1998).

In the present work, supplementation of antioxidants (alone/in combined concentration) resulted in an improved rate of sperm motility in the nicotine-treated samples. Trolox, alone and/or in combination with glutathione caused a better result in increasing the percent motile sperm cells in the samples. Interestingly, combination of trolox and GSH could increase the percent motility rate as much as the control values in the samples treated with 0.5 and 1 mM nicotine concentrations. From the present results, it is further suggested that a combination of 0.02 mM trolox and 5mM glutathione may work out still better. If the combined concentration is administrated to a
sample from a patient (infertile or subfertile due to enhanced ROS production), it should safeguard the cells against oxidative insult and subsequent loss of motility.

During the present investigation a stimulation in the spermatozoal motility rate was observed significantly upon incubation with various concentrations of caffeine (5, 7 & 9 mM). Elevation in the percent motile sperm cells was started at 10 min of incubation time and lasted till 120 min with 5, 7 & 9 mM caffeine concentrations. It was clearly observed that the increasing effect of 7 mM caffeine on the sperm motility was more efficient than those of other concentrations. Caffeine substantially stimulated the sperm cells whose motility had declined because of time and it appears to inhibit the rate of decline with time.

Mammalian sperm motility has been reported as being enhanced and/or prolonged in the presence of caffeine as a phosphodiesterase inhibitor (Levin et al., 1981; Babcock et al., 1983; Fayed, 1996; Lopez and Alvarino, 2000). Treatments with 0.01-20 mM concentrations of caffeine were found to shift the percentage of non-hyperactive sperm cells to either a transitional or hyperactivated state from cryopreserved semen samples (Stachecki et al., 1994). Methylxanthines have been used to increase the motility of freshly ejaculated spermatozoa (Barkey et al., 1984; Aitken et al., 1986; Hammit et al., 1989). It has been demonstrated that caffeine at μM (Coscioni et al., 2001) and/or mM concentration (Lopez and Alvarino, 2000) is able to enhance the frequency and motility rate of sperm cells in mammals. There is a discrepancy therefore, in using the effective dose of caffeine in causing the stimulation of sperm motility in the literature. Caffeine was effective in increasing the sperm motility of cryopreserved samples at 5mM concentration. The motility parameters like curvilinear velocity and lateral head displacement were significantly enhanced (Sharma et al., 1996). In the work by Moussa (1983), it has been shown that at concentrations of 3 and 6 mM, caffeine significantly increased the percent motile sperm cells. In dose >6mM, caffeine exhibited...
Discussion

non-insignificant stimulation that was reversed later on into an inhibitory effect on all the parameters of sperm motility at a dose of 60 mM. At a dose of 120 mM, caffeine caused a complete immobilization of human spermatozoa.

It is well documented that caffeine triggers the activation of some key enzyme like protein tyrosine kinase in sperm cells which is responsible for protein tyrosine phosphorylation of some proteins in the principal region of sperm tail (Pavlok et al., 2001), resulting in the maintenance and increase of flagellar movement and sperm motility rate.

In the current study, caffeine increased the percent motile sperm cells, and the motility was changed from the non-progressive “twitching” type to progressive motility with forward drives. Even though the objective velocity measurements were not performed in our work, the latter observation was unmistakable because only non-progressive motility was found in the unstimulated spermatozoal samples and only active movements was registered in the caffeine-stimulated spermatozoal samples. It is noteworthy that, in the present work the motility stimulation by caffeine was maintained over 120 minutes of incubation which is in agreement with the report in which the caffeine-induced progressive motility lasts for a few hours (Makler et al., 1980). This point has been used as an encouraging sign for the practical use of caffeine to improve the quality of poor semen samples (Prins and Ross, 1985).

In the caffeine (5-9 mM) treated sperm samples, supplementation of antioxidants resulted in a further increase in the motility rate of sperm cells. Trolox, alone and/or in synergistic effect with glutathione was found to be the most efficient antioxidant in elevating the rate of sperm motility in caffeine-treated series. Antioxidant could safeguard the sperm cells against detrimental impact of possible pre-existing and/or caffeine-induced free radicals/lipid hydroperoxides in the sperm plasma membrane. Free radical-absorbing capacity of used antioxidants helped the stimulating aspect of caffeine on sperm cells to maintain properly the sperm motility at a satisfactory level, via either quenching the formed free radicals while the
sperm cells are activated or alleviating the caffeine-induced free radical formation in the spermatozoal samples.

**Sperm Energy Metabolism**

An impaired energy metabolism in spermatozoa may be a potential cause of infertility and therefore, in past some inhibitors of sperm energy metabolism (e.g. α-chlorohydrin) had been found as effective male contraceptives in animals (Ford and Waites, 1986). The concentration of ATP in human semen has been reported to be an index of fertility (Comhaire et al., 1983) although its predictive power was less. Human spermatozoa can obtain energy both from glycolysis (Peterson and Freund, 1970), and to a much lesser extent by mitochondrial oxidative phosphorylation (Ford and Harrison, 1981), but in comparison to other species they drive a greater share of their ATP production from the former (Ford and Rees, 1990). They use ATP to provide energy for motility, to maintain ionic gradients, and as a source of cAMP for different purposes. It has been shown that the ATP concentration of an ejaculate is positively correlated with the concentration of sperm cells and their motility (Irvine and Aitken, 1985).

Comhaire et al. (1983) reported that a positive correlation between the concentration of ATP per milliliter of fresh ejaculate and the percentage of zona-free oocyte penetrated by the sperm cells was established. They also substantiated a positive correlation between the relative fertility in vivo (percentage of pregnancies) and mean of ATP concentration of a group of donor's ejaculates. In bull and boar spermatozoa, the increased motility is accompanied with increase in the rate of glycolysis and of mitochondrial respiration, together with a decrease in the concentration of ATP and rises in ADP and AMP concentrations (Garbers et al., 1973). In bull spermatozoa, the decrease in the cytoplasmic ATP/ADP ratio is the principal factor which controls the increase in the rate of oxidative phosphorylation (Halangk et al., 1987).
Zaneveld (1982) has described that the motility of spermatozoa is regulated by the activity of its mid-piece enzymes which include the enzyme systems involved in glycolysis, the citric acid cycle and oxidative phosphorylation. These enzyme systems, besides the acrosomal enzymes, can be used for the development of new lines of contraceptives. The spermatozoa have the enzymatic machinery for carrying out glycolysis. The glycolysis is catalyzed by the consecutive action of a group of eleven enzymes, which can be easily extracted in soluble form from the cells. These enzymes may be loosely associated with the plasma membrane, myofibrils, and mitochondria (Sidhu and Guraya, 1985). The glycolysis in mammalian sperm cells is primarily determined by the relative concentrations of ATP, ADP, and AMP. The effect of these nucleotides on the sperm metabolism can be expressed in terms of a concept known as the energy charge (EC) which defined as follows:

\[
EC = \frac{(ATP) + \frac{1}{2}(ADP)}{ATP + ADP + AMP}
\]

when EC is reduced, i.e. ATP levels decreased, the glycolysis is increased, because these enzymes are ordinarily inhibited by ATP concentrations (Sidhu and Guraya, 1985). The stimulation of motility of human sperm cells by stimulators like caffeine is accompanied with a fall in the EC value and a marked elevation in the glycolysis rate (Peterson, 1976). Travis et al. (2001) hypothesized that glycolysis is organized in the fibrous sheath of the sperm flagellum to provide ATP to dynein ATPases that generate motility and to protein kinases that regulate motility. Recently, it has been substantiated that, glycolytic ATP production is required for vigorous motility and hyperactivation of human sperm cells (Williams and Ford, 2001). The present investigation was designed to study the effect of nicotine (0.5 and 1mM) and caffeine (5,7 and 9mM) on the activities of certain key enzymes in the energy forming cycle in human sperm cells with antioxidant supplementation (singly/in combination) namely, 1mM ascorbate or 5mM GSH or 0.02 mM
trolox. It is evident that nicotine and caffeine when added to the incubation media containing human sperm cells, caused an inhibition and a stimulation, respectively, in general of all the enzymes studied in the present work. The enzyme activity modulation effect is more pronounced at the higher concentration of the drugs tested.

Our results revealed that the activity of glucose-6-phosphatase upon nicotine treatments (0.5 and 1 mM) was found to be lowered non-significantly (p>0.05) by about 10.14 and 23.74%, respectively. On the other hand, addition of caffeine (5, 7 & 9 mM) resulted in a non-significant elevation in the enzyme activity by about 9.15, 14.43 and 17.06%, respectively. Glucose-6-phosphatase is a terminal hydrolytic enzyme for glycolytic pathway. Inhibition of this key enzyme can lead to the accumulation of glucose-6-phosphate instead of breaking down to the glucose and phosphate moiety. It must be kept in mind that glucose-6-phosphate is also a key hydrolytic enzyme for both glycogenolysis and gluconeogenic pathways and, therefore an inhibition of its activity may more likely to block the production of glucose from other sources such as amino acids, propionate, and oxaloacetate. Altogether, any cause in the flux of carbon through the process of glycolysis can lead to an insufficient energy from the carbohydrate source resulting in infertility. Dawra and Sharma (1985) reported that lipid peroxidation could inactivate the glucose-6-phosphatase which was reversed by antioxidants supplementation. It was determined that spermatozoal ATP levels after ROS treatment were reduced approximately eight-fold in 30 minutes of incubation time (Armstrong et al., 1999). Antioxidant supplementation (singly/in combination) resulted in an elevation in the glucose-6-phosphate activity in both nicotine and caffeine-treated samples non-significantly (p>0.05). It may be suggested that nicotine-induced peroxidation could partially inhibit the enzyme activity, which may be due to the less sensitivity of the enzyme to the peroxidation process. However, caffeine caused a little elevation in the enzyme activity which can be translated into an increase in sperm movement.
Hexokinase as a rate-limiting enzyme in the glycolysis cycle is strongly bound to the mid-piece of spermatozoa (Sidhu and Guraya, 1985). Glycolytic ATP production is required for vigorous motility and hyperactivation in human sperm (Williams and Ford, 2001). The results from the work by Bajpai et al. (1998) indicated that spermatocytes have a greater activity of glycolytic and pentose phosphate pathway enzymes than in spermatids. They showed a more content of hexokinase and LDH plus phosphofructokinase in spermatocytes. It is also reported that the fertilizing spermatozoa are responsible for an increase in both glycolytic and pentose phosphate pathway activity in oocytes during fusion and/or decondensation (Umer and Sakkas, 1999). Also, an isoform of hexokinase (HK1-SC) is localized to fibrous sheath of sperm flagellum (Travis et al., 2001). Our results indicate that nicotine treatments (0.5 and 1 mM) caused an inhibition in the hexokinase activity by 23.04 and 32.28% (p<0.01). Additions of caffeine (5-9 mM) resulted in an elevation (p<0.05) in the enzyme activity (14.35, 16.00 & 19.80%, respectively). Antioxidant supplementation in single form could not register any significant change in the hexokinase activity when compared to the respective drug-treated samples. When combined concentration of antioxidants were used, an elevation in the enzyme activity of nicotine-treated samples was observed as in the case of the addition of ascorbate + trolox and trolox+GSH to the spermatozoal homogenates. No change was observed after addition of combined antioxidants in caffeine-treated samples. It may be suggested that any change in the hexokinase activity by nicotine and/or caffeine, may lead to a greater change in the sperm energy reservoir to maintain its functions. Human spermatozoa can adjust their metabolic performance effectively to meet changes in the energy demand but the glycolytic pathway is more responsive than mitochondrial respiration, at least in terms of the relative changes in the flux of substrates (Rees et al., 1990). It may be speculated from these results that, hexokinase activity is very...
responsive and sensitive to oxidative stress induced by potent oxidants like nicotine.

Glucose-6-phosphate isomerase activity was found to be lowered upon nicotine treatments (0.5 & 1mM) by 17.37 and 27.55% (p<0.05 & p<0.01), respectively. On the other hand, an elevation in the enzyme activity was observed following caffeine treatments (5-9 mM) by about 7.90, 20.19 & 32.53% (p>0.05, p<0.05 & p<0.01), respectively. An inhibition in the glucose-6-phosphate isomerase may lead to the reversal of glycolysis by blocking the formation of fructose-6-phosphate and then to fructose 1-6, diphosphate in the sperm cells which could result in a lower stage of energy supply. Dawra and Sharma (1985) revealed that peroxidation can be a cause of inactivation of glycolysis cycle in sperm cells. Caffeine as an intracellular calcium increasing agent could elevate the rate of enzyme activity in a dose-dependent fashion. In this connection, it has been substantiated that increased intracellular calcium concentration in ram spermatozoa had an inhibitory effect on mitochondria-dependent motility but not such effect observed on glycolysis-dependent motility (Breitbart and Nass-Arden, 1995). Energy metabolism of boar spermatozoa is typically based on glycolysis consuming extracellular carbohydrates and producing lactate and protons (Kamp et al., 1996). In rats, spermatozoa with the greatest glycolytic potential and lowest TCA cycle activity appear to be programmed to utilize exclusively glucose/fructose for energy (Bajpai et al., 1998). Antioxidant supplementation (singly) could register a significant alteration in enzyme activity in both the nicotine and caffeine-treated samples, using a combined concentration of antioxidants (trolox+GSH) resulted in an elevated rate of enzyme activity in nicotine-treated samples and also in 7mM caffeine-treated ones. The combination of trolox and glutathione may exert a synergistic effect with caffeine in the treated series. The peroxidative damage to enzyme may be reversed by the effect of the same antioxidant combination in the nicotine-treated samples.
Lactate dehydrogenase (LDH) catalyzes the reversible conversion of lactate to pyruvate in the presence of NAD⁺. Kaur and Guraya (1981) have described a moderate activity of LDH within the seminiferous tubules using histochemical techniques. The observed enzyme activity was reduced after treatment with α-chlorohydrin. LDH activity was also significantly decreased after gossypol treatment when observed by Vyas et al. (1985). LDH has been shown to play an important role in the mitochondrial lactate-pyruvate shuttle system, to transfer reducing equivalents from cytosol to mitochondria for the generation of energy necessary for motility and survival of germ cells (Blanco et al., 1980). A greater rate of LDH activity was also reported in the spermatocytes than in the spermatids in rat (Bajpai et al., 1998). Our data has shown that nicotine (0.5 & 1mM) additions caused a lower rate of activity for LDH by 14.45 & 28.00% (p>0.05 & p<0.05, respectively). On the other hand, caffeine (5-9mM) treatments caused an elevated rate of activity for LDH by 19.74, 31.10 & 32.90% (p<0.05, p<0.05 & p<0.01), respectively. Fahmy and Walsh (1955) substantiated the decreased LDH activity with higher concentrations of nicotine. Chronic exposure to constant light promotes a reduction of fertilizing ability in rats which indicates that continuous lighting reduces the total LDH activity, possibly due to moderate aging of spermatozoa within the duct by lengthening the sperm transit through the epididymis (Ponc et al., 2001). Antioxidant supplementation (singly/in combination) did not register a significant alteration in the activity of human sperm LDH in the drug-treated samples. It may be suggested, at least in nicotine-treated ones, that the peroxidation induced by nicotine was not reversible and resulted in a severe damage to the enzyme structure as well as function. In human spermatozoa Rees et al. (1990) reported that, addition of caffeine resulted in a very significant increase in the rate of glycolysis but a smaller, less consistent increase in the oxidation of lactate or pyruvate to CO₂ follows. The increased flux through the glycolytic pathway provides a substantial proportion of the additional ATP required to sustain an increased motility.
Glucose-6-phosphate dehydrogenase (G6PDH) is the first enzyme in the pentose phosphate pathway (PPP) was found to be inhibited by the effect of nicotine (0.5 & 1mM) treatments (p<0.05 & p<0.01, respectively). The rate of G6PDH activity was found to be elevated in caffeine (5-9mM) additions (p<0.05, p<0.01 & p<0.05, respectively). There is a close link between PPP and glycolytic pathway to provide sufficient energy as required. In agreement to our results, Griveau _et al._ (1995) substantiated that under oxidative stress conditions, the activity of G6PDH is inhibited significantly which shows the enzyme is very vulnerable to oxidative stress in spermatozoal samples (Oliver _et al._, 1987). In contrast, Ferrandi _et al._ (1990, 1995) reported that lipid peroxidation increases NADPH production from PPP by the increased activity of G6PDH in mouse spermatozoa. G6PDH influences the concentration of GSH through production of NADPH as a reducing co-factor to glutathione reductase. Inactivation of G6PDH could result in a decrease in the antioxidative protection exerted by the glutathione-dependent system. Umer and Sakkas (1999) substantiated that fertilizing sperm is responsible for an increase in both glycolytic and PPP activity in oocytes during fusion and/or decondensation. In the present study, antioxidant supplementation (singly) could reverse the inhibition of G6PDH in nicotine-treated samples, as the best result was obtained with the addition of trolox. Antioxidant combinations when added to nicotine-treated sample could exert an improving effect on the activity of G6PDH which the best result was obtained with a combination of trolox and glutathione. Antioxidant supplementation (singly/in combination) could not exhibit a significant increasing influence on the activity of G6PDH in caffeine-treated samples, expect in 7mM caffeine treatment. However, addition of combination of trolox and GSH resulted in a further (p<0.05) increase in G6PDH activity of the same samples.

**Sperm Morphology**

The ejaculated human spermatozoa studied during the course of present investigation revealed typical ultrastructural morphology of the...
mammalian sperm cells (Hafez and Kanagawa, 1973; Mann and Mann, 1981). Incubation of human ejaculated spermatozoa with a high concentration of nicotine, 1mM, showed various morphological abnormalities under SEM. In addition to the coiling of the tail, severe rupturing and vesiculation of plasma membrane in both the acrosomal and post-acrosomal regions of head as well as narrowing of mid-piece and abaxial tails of the human spermatozoa were registered. The membrane rupturing was maximum in the anterior part of the sperm head. These results are supported by large number of earlier findings of Vogel et al. (1979), Evans et al. (1981), Handelsman et al. (1984); Vogt et al. (1986). All of them are of the view that the cigarette smokers have a higher frequency of morphologically abnormal sperm than non-smokers. Rubes et al. (1998) has described that cigarette smoking elevated the frequencies of round-headed sperm cells in teenage men. The authors also revealed that the regression between concentration of cotinine (a major metabolite of nicotine) in urine and the proportion of round-headed spermatozoa was significant. During the course of present investigations however this round head morphology was not observed. This round-head morphology has been associated with an increased risk of infertility (Saaranen et al., 1989).

Vine et al. (1994, 1996) reported that cigarette smoking is associated with lowered semen quality. It has also been reported that an increase in abnormal sperm morphology was related to the dose of cigarette smoking (Evans and Fletcher, 1981; Merino et al., 1998). Pekarsky et al. (1995) substantiated that the rate of sperm penetration into the oocyte was more significantly decreased with the increase in nicotine concentrations (>0.1mM). This might be a result of nicotine-induced alterations in the sperm morphology, thereby lowering the rate of sperm penetration.

Recently, Wong et al. (2000) demonstrated a statistically significant correlation between cotinine concentration in the seminal plasma of smokers and the percentage of abnormal sperm morphology. Kanwar et al. (1993) reported disruptive changes such as rupturing and splitting of outer plasma
membrane of human sperm cells as well as cytoplasmic blebs following treatment with nifedipine, a calcium channel blocker. Since, antioxidant supplementation resulted in a better preservation of morphology for sperm cells, it can be suggested that the free radical-induced lipoperoxidation was the major cause for resultant abnormalities in the nicotine-treated samples during the present work. Collectively, our results and other reports on the impact of nicotine and/or cigarette smoking on the sperm morphology suggest that nicotine as a major part of cigarette smoke is able to alter some aspects of sperm morphology and its functional ability towards male infertility.

It has been shown that treatment of sperm cells with 9mM caffeine for 120 minutes caused a disruption of the sperm head and swelling of mid-piece (Harrison *et al*., 1980). Barkey *et al.* (1984), on the other hand, demonstrated that addition of 7.2 mM caffeine to the sperm sample from fertile donors did not register a significant toxic effect on the sperm morphology. Though they observed some types of abnormalities in the sperm samples such as acrosomal malformation, abaxial tail and deformities in the neck region. Our results reveal that, addition of 9mM caffeine exerted minor defects and disruption in the sperm morphology, including slight vesiculation in the post-acrosomal region, coiling in the tip of sperm tails and abaxial tails. The caffeine-induced minor morphological alterations lead us to conclude that this agent has much less damaging impact on sperm morphology and its functional ability to fertilize ovum. Also, an improvement was observed after supplementation of antioxidants in sperm morphology of caffeine-treated spermatozoal samples. The presence of these abnormalities in both the nicotine and caffeine-treated sperm samples indicate that these agents may have some spermicidal effects particularly in the case of nicotine and their mode of action involve major membrane damage.

**Sperm DNA Integrity**

Reactive oxygen species (ROS) have been found to have dual effects on the human spermatozoa. Physiologically, ROS are required for the
capacitation process, but at high concentration, on the other hand, ROS can induce loss of motility and lead to the sperm dysfunction, including poor sperm-zona pellucida binding and sperm-oocyte fusion (Aitken et al., 1989; De Lamirande and Gagnon, 1993; Aitken and Fisher, 1994; Oehninger et al., 1995; Irvine et al., 2000). Recent studies have also shown that, ROS may attack DNA integrity of human sperm cells. Generation of ROS induced by NADPH and xanthine/xanthine oxidase system or by direct addition of \( \text{H}_2\text{O}_2 \) to the sperm samples was shown to cause a significant increase in DNA fragmentation (Aitken et al., 1998; Lopez et al., 1998). Furthermore, fragmentation of sperm DNA was shown to be inversely correlated with semen quality, particularly sperm morphology and motility and fertilization rate after *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Sun et al., 1997; Lopez et al., 1998; Irvine et al., 2000; Muratori et al., 2000; Host et al., 2000). Spermatozoa have only two defence mechanisms against oxidative attack to their DNA; the packing arrangement of DNA, and the seminal plasma. During spermatogenesis, the chromatin becomes highly condensed within a protamine matrix (Sidney and Grimes, 1986). The sperm DNA is organized into the loop which is anchored to the base of the sperm tail by the nuclear annulus and stabilized by disulfide bonds (Barone et al., 1994). This tight packing of the DNA reduces exposure to free radical attack. The second line of defence is the antioxidant capacity of its seminal plasma (Lewis et al., 1995, 1997). Baseline DNA integrity in spermatozoa is lower than that of somatic cell types (Hughes et al., 1996, 1997), possibly a reflection of its physiological role in the fertilization (Singh et al., 1989). On the other hand, spermatozoa produced by infertile men were shown to have DNA which was more susceptible to damage by irradiation than that from fertile men (Hughes et al., 1996). The capacity for DNA repair is lost as the mature sperm cells do not have any repair enzymes due to discarding the majority of their cytoplasm during the final stages of spermatogenesis (Van Loon et al., 1991). DNA
damage may not prevent fertilization from occurring but may lead to fetal abnormalities only be apparent later (Hughes et al., 1996).

The comet assay has been used to examine the effect of various chemicals, such as food mutagens on spermatozoa in vitro, and may prove to be a very sensitive test to examine the DNA strand breaks in the sperm chromatin (Anderson et al., 1997). Single-stranded lesions in the sperm DNA should be repaired in the oocyte upon fertilization and should not be lethal. However, if a fertilizing spermatozoon possesses single-stranded DNA breaks of significant size, they may prove difficult for the oocyte to repair and may lead to failure in either the fertilization process or later in the development (Sakkas et al., 1999). Double-stranded breaks are considered to be more lethal lesions to the sperm DNA than single-stranded ones (Kizilian et al., 1999). In the present study, acridine orange stain was used to distinguish the single-stranded from double-stranded DNA breaks. Acridine orange can bind to the double-stranded DNA and fluoresces green but when binds to the single-stranded DNA breaks, it fluoresces red (Tejada et al., 1984; Zini et al., 2001). Our results indicate that, almost all the comet tails were double-stranded and some single-stranded DNA breaks were also observed in both nicotine and caffeine-treated samples. It may be speculated that when nicotine and caffeine are present in the sperm environment at high concentration, may induce double-stranded breaks/mutagenic impairments in the sperm DNA towards genetic alterations in the offspring. Lopes et al. (1998) demonstrated that men with a sperm population containing more than 25% DNA damage are more likely to experience a fertilization rate less than 20% after ICSI technique.

Nicotine as a potent oxidative agent could induce DNA damage with a percentage more than of caffeine as a mild oxidant in the spermatozoal samples as judged by the presence of more detected comets in the samples. Both ROS generation and oxidative DNA base damage are elevated in the spermatozoa of infertile men (Aitken et al., 1992; Kodama et al., 1997; Barroso et al., 2000). It may be suggested that ROS production and subsequent
lipoperoxidation can be selected as predictors of the DNA damage in the spermatozoal samples. The semen of heavy smokers is characterized by increased level of oxidative DNA base damage, high chromatin fragmentation and low concentration of antioxidants (Fraga et al., 1996). As a consequence, the offspring of such subjects are four to five times more likely to develop childhood cancer than the children of non-smoking fathers (Ji et al., 1997; Sorahan et al., 1997). Shi et al. (2001) reported that cigarette smoking may increase the risk of aneuploidy for the certain chromosomes in both light and heavy smokers. It was also substantiated that human sperm chromatin becomes cross-linked under the oxidative stress and exhibits increased DNA strand breaks (Twigg et al., 1998). Smoking is associated with an increase in strand breaks in the sperm DNA structure as a result of oxidative stress (Potts et al., 1999). Jafari and Rabbani (2000) have also reported a sharp increase in the superoxide anion formation and DNA degradation following addition of 5 to 20 mM concentrations of caffeine to the culture medium of macrophages derived from rat lung.

As antioxidants are compounds which scavenge and dispose the ROS in vitro and in vivo, depriving sperm of the antioxidant protection available in the seminal plasma may lead to the DNA damage, while protection can be provided by supplementing sperm sample with antioxidants such as ascorbate and alpha tocopherol (Hughes et al., 1998). Seminal plasma from infertile men shows a lower antioxidant capacity than that of fertile subjects (Lewis et al., 1995, 1997). Our data reveal that antioxidant supplementation can improve the DNA integrity and protect sperm DNA from nicotine and caffeine-induced free radical formation as judged by the lower percentage of sperm cells having comet appearance. This effect was prominent in caffeine-treated spermatozoal samples than in the nicotine-treated ones which reversed the DNA damage to the control level when antioxidant combination was added. The presence of about one percent sperm cells with comet appearance in the control samples may be attributed to the endogeneous DNA damage from the spermatogenesis.
process. The combination of trolox and glutathione was found to produce better result in lowering the amount of DNA breaks. Fraga et al. (1991) and Lewis et al. (1997) reported that ascorbate could protect spermatozoa from DNA damage. Alpha tocopherol (Trolox) has also been shown to provide protection against DNA damage to the sperm cells (Fraga et al., 1996; Hughes et al., 1998). Addition of ascorbate and/or alpha tocopherol to the sperm preparation did not affect the baseline DNA integrity but could provide sperm with complete protection against H$_2$O$_2$-induced DNA breaks (Donnelly et al., 1999). Addition of seminal plasma as a rich source of natural antioxidants to the sperm preparation resulted in a significant decrease in the DNA strand breaks and TBARS production (Potts et al., 2000). In disagreement with Hughes et al. (1998) which found the addition of combination of ascorbate and trolox to the spermatozoal samples could result in more DNA strand breaks, we observed that this combination can afford satisfactory protection to the sperm DNA against ROS. It may be suggested that there is a narrow physiological range in which antioxidants can work synergistically to act against the oxidative conditions.