Introduction
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Infertility in males may result from several primary biological impairments such as semen abnormalities or hormonal alterations (Baird and Wilcox, 1986). Male reproductive functions may also be altered by chronic exposure to bioactive compounds, capable of crossing the blood-testis barrier following systemic absorption. Certain chemical and physical agents may also have either beneficial or detrimental influences on sperm motility, rate of respiration, glycolysis, maturation, capacitation and metabolism (Guraya, 1987). Male infertility accounts for 40% of infertility problems (Fleming et al., 1995) both for primary and secondary reasons. Until now however, no appropriate treatment for idiopathic male infertility has been found: neither have any controlled, double-blind studies demonstrated any significant improvement in pregnancy rates following the use of hormones and/or hormonal analogues (Nieschlag and Leifke, 1997). In addition to the endocrine aspects a large number of toxicological substances and pharmacological and physical agents (e.g. radiation damage) can cause reproductive intervention of the cellular and molecular level. Among these, the influence of active oxygen derivatives and related antioxidant treatments has become of increasing interest in recent times (Rolf et al., 1999).

Infertility may also be linked to the DNA damage, as the sperm DNA of infertile patients has been shown to be more susceptible to damage in vitro than from fertile men (Hughes et al., 1996). A major source of damage to DNA is by reactive oxygen species (ROS) (Steenken, 1989; Dizdaroglu, 1992; Palomba et al., 1996). Molecular oxygen (O₂) supplied at concentrations greater than those in normal air has long been known to damage plants, animals and aerobic bacteria such as Escherichia coli. Plots of the logarithm of survival time against logarithm of the oxygen pressure have shown an inverse, approximately linear relationship for protozoa, insects, fish, mice, rats and rabbits. Indeed, there is considerable evidence that even 21% O₂ has slowly
manifested the damaging effects on the survival of the organism. The effects observed vary considerably with the type of organism used, its age, physiological state and diet, such as the presence in the diet of varying amounts of vitamins E and C, transition metals, antioxidants (now added to many animal and human food in routine) and polyunsaturated lipids (Halliwell and Gutteridge, 1984).

In human semen, defective sperm cells and contaminating neutrophils are potential sources of the oxidative agents/ROS e.g. hydrogen peroxide (Aitken et al., 1992; Kessopoulou et al., 1992; Gomez et al., 1996). Spermatozoa are uniquely susceptible to oxidative damage because of their stage of differentiation (Jones et al., 1973). One feature of the semen of infertile men, particularly those with oligoasthenozoospermia is the production of excessive levels of ROS (Aitken et al., 1989; 1991).

During the past decade evidence has accumulated to support a pivotal role of ROS in the pathogenesis of sperm dysfunction among men with infertility. Increased generation of ROS has been documented in the sub-fertile men with varicocele (Sharma and Agarwal, 1996), with immunological infertility, with idiopathic oligozoospermia, and in cases with excess concentration of white blood cells in semen (Zalata et al., 1995). In addition, certain lifestyle factors such as tobacco-smoking and environmental agents may reduce the antioxidant capacity of seminal plasma and impair the secretion of the accessory sex glands (Pakrashi and Chatterjee, 1995; Depuydt et al., 1996; Klinefelter and Hess, 1998). Due to their paucity in cytoplasm, spermatozoa have little defense against oxygen damage and are highly sensitive to ROS. It may change the fatty acid composition of phospholipids of the sperm membrane, and induce oxidative damage to sperm DNA. The former effect in particular results in a decreased proportion of polyunsaturated fatty acids and increased mean melting point, corresponding to the decreased fluidity of the membrane (Comhaire et al., 2000).
One consequence of excessive ROS generation is the peroxidative damage to the sperm plasma membrane, which leads to an impairment of sperm function that is reflected in decreased pregnancy rates in vivo as well as in impaired fertilization in vitro (Aitken et al., 1991; Sukcharoen et al., 1996). ROS can modify sperm cytoskeleton and axoneme development (De Lamirande and Gagnon, 1992a). Peroxidation in sperm plasma membrane, however, can occur spontaneously and is greatly enhanced in human subfertile ejaculates (Alvarez and Storey, 1989; Aitken et al., 1993) but the effects induced by ROS are further modulated by the nature and the amount of ROS involved as well as by the moment and the length of ROS exposure (De Lamirande and Gagnon, 1992a; Griveau and Le Lannou, 1997). Physiologically, ROS may also participate in the signal transduction processes such as in the pathway of programmed cell death (Polyak et al., 1997). It is well documented that ROS could be involved in two main functions of sperm cells, i.e. their kinetic function, which enables them to migrate through the female genital tract and across the cumulus cells, and their fusigenic function, which enables them to bind to the zona pellucida and then fuse with the oocyte membrane (Aitken et al., 1989; Bize et al., 1991; Griveau et al., 1995; Kodama et al., 1995). Collectively, whenever ROS are found in low concentrations, they act as mediators of normal sperm function, whereas whenever they are produced in excess, they prove to be highly toxic to the cell.

Oligozoospermia (a sperm count of less than $20 \times 10^6$) is found in 16 - 41% of infertile couples. The primary difference between oligozoospermic specimens and normal fertile controls is in the relative contribution of the spermatozoa to the ROS generating capacity of the ejaculate. In oligozoospermic patients, the spermatozoa are the predominant source of ROS and generate extremely high levels of ROS compared to those produced by spermatozoa from normal fertile men (Sharma and Agarwal, 1996).

A complex antioxidant system is present in spermatozoa and seminal plasma to scavenge the oxygen radicals and prevent their damaging action.
under normal physiological conditions. Such a system embraces enzymatic activities, such as superoxide dismutase, catalase and glutathione peroxidase, and also non-enzymatic antioxidants/scavengers e.g. water soluble (ascorbate, glutathione and uric acid) and/or fat-soluble (vitamin E, carotenoids and ubiquinones) natural compounds (Doba et al., 1985; Chow, 1991; Dawson et al., 1992; Surai et al., 1998).

It has been shown that seminal plasma from infertile men has a significantly lower total antioxidant capacity than that from fertile men (Lewis et al., 1995). Recently, there has been much debate regarding the potential advantage of antioxidant therapy in improving male fertility (Tarin et al., 1998; Lenzi et al., 1998). The discovery, that lipid peroxidation is a causative mechanism in the aetiology of defective sperm function, is important because it leads logically to the use of antioxidants as a strategy to reverse the damage caused by oxidative stress (Aitken and Clarkson, 1988). An effective antioxidant can be successfully used to reverse the oxidative damage inflicted on human spermatozoa during sperm preparation procedure that involves centrifugation of ejaculated cells in the absence of protective environment normally provided by seminal plasma (Aitken, 1994; 1995).

Aims and Objectives

The present study has been designed with the aim of establishing the peroxidative damages and impairment of cellular functions by caffeine and nicotine in the human ejaculated spermatozoa. The major focus of attention has been laid to investigate the effectiveness of antioxidants namely ascorbic acid (AA), glutathione (GSH) and trolox, a water-soluble analogue of α-tocopherol. An attempt has been made to achieve the following objectives so as to contribute to the understanding of the altered biochemical and physiological processes in the sperm cells under various experimental conditions:
1. Measurement of thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation (LPO) and membrane integrity of the human ejaculated spermatozoa:
   a. In the presence of different concentrations of the drugs, caffeine and nicotine, and the antioxidants such as vitamin C, glutathione and trolox
   b. In the presence of ferrous sulphate (FeSO₄) as a potent oxidant, with/without supplementation of the selected concentrations of antioxidants
   c. In the presence of various concentrations of caffeine and nicotine with supplementation of selected concentrations of antioxidants
   d. In the presence of caffeine and nicotine, and supplemented with different concentrations of LPO-inducing ferrous-ascorbate (Fe²⁺-AA) system.

2. Extraction and analysis of the spermatozoal lipids in membrane suspensions in the drug treated-antioxidant supplemented series.

3. Characterize the activity of certain components of γ-glutamyl cycle in the drug treated-antioxidant supplemented spermatozoal samples.


5. Evaluate the activity of some enzymes of energy metabolism in human spermatozoal samples upon drug treatment and antioxidant supplementation.

6. Examine the functional ability of human ejaculated sperm cells such as the motility in different conditions of drug treatment with antioxidants.

7. Evaluate the effect of drug treatment and antioxidant supplementation on ultrastructural morphology by the scanning electron microscopic technique.
8. Evaluate the effect of drug treatment and antioxidant supplementation on the integrity of human spermatozoa DNA by the single cell gel electrophoresis (comet) assay.

Collectively, the need for the present study was the assessment of the value of antioxidants in protecting against oxidative stress and resulting processes so that they might be used in the media for sperm preparations and also for the techniques during *in vitro* fertilization (IVF) and/or intrauterine insemination (IUI), and thus prove beneficial for the cryopreservation techniques.