Abstract
Spermatozoa were the first type of cells reported to produce free radicals. Reactive oxygen species (ROS) mediated damage to sperm is a significant contributing factor to male infertility. Impaired motility, impaired fertilization and oxidative DNA damage are three inter-related mechanisms that account for oxidative stress mediated male infertility. Spermatozoa lack cytoplasmic antioxidant defense due to exclusion of cytoplasm and therefore rely upon antioxidants present in the seminal plasma. Centrifugation of a semen sample prior to its use for intra-uterine insemination (ICI) and in vitro Fertilization (IVF) induce oxidative stress. Therefore there is need to supplement the semen with antioxidants. In the present investigation attempts were made to study the effects of in vitro supplementation of non-enzymatic antioxidant ascorbic acid on sperm plasma membrane integrity, acrosome intactness and mitochondrial activity index. There was highly significant (p<0.001) improvement in these parameters that relate to healthy state of the spermatozoa.

Key words: Spermatozoa, Oxidative stress, Male infertility, Antioxidants, Ascorbic acid

Introduction
Spermatozoa is equipped with poor antioxidant defense system as compared to other cells. Among environmental, genetic and physiological factors responsible for the poor sperm function, free radical induced oxidative stress gained much attention, due to its deleterious effects on sperm plasma membrane and DNA damage leading to infertility (1-3). Though having essential role in sperm physiological processes such as capacitation, hyperactivation and sperm-oocyte fusion (4-5) spermatozoa are sensitive to Reactive Oxygen Species (ROS) because they lack cytoplasmic defenses (6). Moreover sperm plasma membrane is rich in polyunsaturated fatty acids, which are vulnerable oxidative damage (7). Contaminating leucocytes & immature spermatozoa are the major sources of ROS in the semen. (3, 8-11). Oxidative damage to plasma membrane, acrosomal and mitochondrial membrane in the form of lipid peroxidation results in the loss of functional membrane integrity and decreased production of ATP, which leads to infertility. Along with membrane damage ROS have found to be responsible for nuclear and mitochondrial DNA damage offering a damaged genome to the next generation at the onset of fertilization (12-13). Antioxidants are the scavenger molecules, neutralizing the damaging effects of free radicals. Ascorbic acid is a well known, non-enzymatic, water soluble natural antioxidant having a capacity to minimize the ROS damage by its chain breaking activity (14).
The present investigation was carried out with the hypothesis that in vitro supplementation of ascorbic acid can provide extra protection to spermatozoan plasma membrane, acrosomal membrane, mitochondrial activity index against the ROS in semen and therefore can improve motility and viability. This is an attempt to provide the direction to Assisted Reproductive Techniques (ARTs) in minimizing the failures resulting from ROS.

Material and methods: Collection of the semen sample

Human semen samples were used in the present investigation. Semen samples from twenty-five healthy volunteers of the age group 22-25 years old were collected by masturbation after sexual abstinence for 2-3 days as per the WHO guidelines 1999 (15). Immediately after liquefaction each semen sample was divided into two groups.

1. Control group: 0.5mL of the liquefied semen was mixed with 0.5mL of the Ringer Tyrode solution (NaCl 0.8g, KCl 0.02g, CaCl₂ 0.02g, NaHCO₃ 0.1g, NaH₂PO₄ 0.005g, MgCl₂ 0.01g, Glucose 0.1g in 100mL double distilled water) and incubated at 37°C for 60 min.

2. Experimental group: 0.5mL of the liquefied semen was mixed with 0.5mL of the Ringer Tyrode solution containing 1mM ascorbic acid (Merck India Ltd). The suspension was incubated at 37°C for 60 min.

Motility: Semen sample from both the groups was analyzed for % motility as per the WHO guidelines 1999 (15).

Viability (16): A drop of semen sample was mixed with a drop of 0.1% Trypan blue on a clean grease free slide and observed for stained and unstained spermatozoa under 400 X magnification. Plasma membrane of the live spermatozoa is impermeable to Trypan blue. Therefore, live spermatozoa remain colourless while dead spermatozoa get stained blue in colour.

Hypooosmotic swelling test (HOS) (1)

0.1 mL of the semen from each group was mixed with 0.9 mL of 150mosmol solution of fructose and sodium citrate. The mixture was incubated at 37°C for 30 min in a tightly stoppered vial. A drop of incubated mixture was placed on a Neubauer’s chamber and allowed to stand for 1 min after placing a cover-slip and observed under dark field microscope at 40 X objective for spermatozoa with coiled tails.

Viability in Hypoosmotic Solution (VHOS) (17)

0.1mL of the semen from each group was mixed with 0.9 mL of 150 mosmol solution of fructose and sodium citrate containing 0.1% Trypan blue and the percentage of unstained spermatozoa with coiled tails was calculated.

Gelatinolysis test for acrosome intactness (18)

10 ÔL of the semen sample was diluted with 190 ÔL of Phosphate buffered saline (PBS) (pH 7.8) containing 3% D-glucose solution. 20 ÔL of this diluted semen was smeared on 5% aqueous gelatin coated slides and incubated at 37°C for 2 hours in moist chamber. The halo diameter was measured under dark field microscope using 40 X objective and with an eyepiece micrometer. The halo formation rate was calculated per slide as the percentage of spermatozoa showing a halo. One hundred spermatozoa were evaluated. An acrosin activity index was calculated by multiplying the halo diameter by the halo formation rate.

Sperm mitochondrial activity index (19)

15 ÔL of semen sample from each group was mixed with 15 ÔL of 0.1% Nitro Blue Tetrazolium (NBT) in Tris-HCl buffer (pH 7.4), mixed well and incubated at 37°C for 30 min.
After incubation, a wet smear of these contents was prepared on a pre cleaned slide. Allowed it to dry and fixed in neutral buffered formalin and observed under 100 X objective for NBT precipitation. On the basis of NBT precipitation in the middle piece of the sperm the spermatozoa were scored for mitochondrial activity index.

**Statistical analysis:** Results were interpreted by using paired t test.

**Results and Discussion**

There was highly significant (p<0.001) increase in percentage of motile and viable spermatozoa in ascorbic acid treated group than the control (Figure 1). Like wise there was highly significant (p<0.001) improvement in percentage of hypoosmotically-swollen spermatozoa, percentage of viable and hypoosmotically-swollen spermatozoa (Figure 2), sperm mitochondrial activity index (Figure 1) and acrosin activity index (Figure 3) in ascorbic acid treated group than the control (Table 1).

The imbalance between generation of free radicals and their inactivation results in oxidative stress a condition resulting in increased rate of cellular damage (7). Damaging effects of ROS as a factor contributing to male infertility have been documented earlier (20). At cellular level, membrane fluidity is the crucial factor responsible for sperm-oocyte fusion reflecting the success of fertilization (21,22). Oxidative damage to mitochondrial membrane lowers the production of ATP (23) ultimately affecting the motility, as there is a positive correlation between ATP production and motility (24). The overall effect of membrane damage might be responsible for continuous decease in motility and viability of spermatozoa after ejaculation (25).

Though having antioxidant property, Rolf et al (26) did not find any significant change in semen parameters in aesthenozoospermic patients with the dietary supplementation of ascorbic acid at the concentration of 1000 mg/day. Fernandez-Santos et al. (27) also found that

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<th>Table 1. Effect of ascorbic acid on various sperm function tests after 60 min of incubation at 37°C (Results are mean ± SEM n=25)</th>
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<tbody>
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<td>Sperm function tests</td>
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<tr>
<td>% motility</td>
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<td>% viability</td>
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<td>Sperm mitochondrial activity index</td>
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<td>% of swollen tail spermatozoa (HOS)</td>
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<td>Acrosin activity index</td>
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***indicates p<0.001

Protective effects of *in vitro* supplementation of ascorbic acid
**References**


8. Wolff, H. and Andeson D.J. (1988). Immunohistologic characterization and


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