Antioxidant levels in blood and seminal plasma and their impact on sperm parameters in infertile men

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Excess reactive oxygen species (ROS) beyond the scavenging capacity of antioxidants leads to DNA damage and oxidation of lipoprotein components at the cellular and subcellular level. The oxidative stress (OS) adversely affects sperm function by altering membrane fluidity, permeability and impairs sperm functional competence. In the present study, the OS status in seminal plasma and blood serum in infertile men and its relationship with spermatozoa parameters have been investigated. Four groups of infertile men viz., oligozoospermic (n = 15), asthenozoospermic (n = 17), teratozoospermic (n = 19), and oligoasthenoteratozoospermic (n = 9), and healthy fertile controls (n = 40) have been analyzed for superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) in seminal plasma and blood serum. Significant correlation between blood serum SOD and sperm count has been observed in patients (p = 0.018) and controls (p = 0.021). Similarly, significant correlation between blood serum GSH and sperm progressive motility in patients (p = 0.036) and controls (p = 0.029) is observed. The low seminal MDA is associated with increase in sperm progressive motility in patients (p = 0.039) and controls (p = 0.028). Positive correlation is found between increased seminal MDA levels and abnormal sperm morphology in both patients and controls (r = 0.523, p = 0.029; r = 0.612, p = 0.034 respectively). Correlations between blood SOD and sperm count and between blood GSH levels and progressive motility suggest that these can be important biochemical markers in assaying the sperm count and motility. A negative correlation of motility with seminal MDA indicates that sperm membrane lipid peroxidation affects the fluidity and thus mobility of sperm axoneme. This affects functional competence of the sperm and acts like a biological safeguard. The results of the present study suggest the prospects of using the blood serum and seminal plasma antioxidants as a valuable tool to evaluate the sperm reproductive capacity and functional competence.

Keywords: Male infertility, Antioxidants, Glutathione, Malondialdehyde, Catalase, Superoxide dismutase, Oxidative stress, sperm parameters, Miscarriage, Abortion

Oxidative stress (OS) is an important pathophysiological factor in the aetiology of idiopathic male infertility. It is produced by increased generation of free radicals as reactive oxygen species (ROS) and reactive nitrogen species (RNS) or by decreased antioxidant production. The fine balance between free radicals and antioxidants is stringently maintained in a normal fertile male and their imbalance manifests itself in the form of various pathological sperm parameters and altered sperm function. Recently, adverse effect of OS on sperm genomic integrity has also been emphasized.

Spermatozoa are highly susceptible to damage induced by OS, because of the high polyunsaturated fat content in the plasma membrane, low concentration of scavenging enzymes within the cytoplasm and higher levels of free radicals. OS induces mitochondrial genome mutations and interferes with normal sperm function via peroxidation of unsaturated fatty acids especially docosahexaenoic acid in the sperm plasma membrane. The lipid peroxides formation affects sperm membrane fluidity and flagellar motion, leading to sperm dysfunction. Mitochondrial mutations impair metabolism, and results in energy crisis in the sperm, thus leading to the increased functional impairment of the sperm.
Although human spermatozoa are known to possess major antioxidant defense systems including CAT, superoxide dismutase (SOD) and glutathione, which neutralize the effects of free radicals, but their effectiveness is impaired by their limited concentration\(^9\). Moreover, the decrease in cytoplasmic volume during spermiogenesis\(^9\) further decreases the antioxidants present in the sperm.

OS is known to impair fertilization, induce cleavage, block and impair embryogenesis. It is also postulated that if diagnosed early by presence of increased MDA levels, administration of antioxidants may not only restore sperm function, but may also prevent irreversible nuclear and mt DNA damage\(^1\). Therefore, knowledge of the antioxidant status and its impact on sperm parameters is critical for providing early and prompt administration of antioxidants to such men. In this study, we have investigated the antioxidant status of blood and seminal plasma and correlation of OS with spermatozoa parameters.

**Material and Methods**

**Patients**

Sixty infertile men and 40 fertile controls were included in the study after informed consent and permission of Institute’s Ethical Clearance Board. These men were referred from Department of Urology, Department of Obstetrics and Gynecology, AIIMS, New Delhi and ART Centre, Army Research and Referral Hospital, New Delhi. The age of patients and healthy fertile controls was between 32.32 ± 2.09 and 28.65 ± 1.69 yrs, respectively. Males with idiopathic infertility and abnormal sperm parameters were included as study group. Males with normal semen parameters and who had fathered a child in last 2 yrs were considered as controls. Exclusion criterion included infertility attributed to obstructive pathology, infection, medication or trauma.

**Semen analysis**

Semen samples were obtained by masturbation and collected into sterile containers after a period of 72-96 h of sexual abstinence. Specimens were allowed to liquefy for 30-40 min at room temperature and conventional semen analysis was performed\(^10\) within 1 h of collection. Sperm concentration was determined by diluting a semen sample in semen dilution fluid [5 g NaCl and 100 ml formaldehyde (40%) made up to 100 ml with distilled water]. Thereafter, some quantity of diluted sample was transferred to neubauer counting chamber and sperms were counted under the microscope. Sperm motility was expressed as the percentage of spermatozoa that showed forward progression (sum of grade a and b sperms). The viability was expressed as percentage of live cells by analyzing minimum 100 spermatozoa after eosine-nigrosine staining. The morphology was expressed as percentage abnormal sperms, including the head, midpiece and tail defects.

Samples were divided into four categories: oligoasthenozoospermic (sperm count less than 20 million per ml), asthenozoospermic (progressive motility less than 50), teratozoospermic (normal sperm morphology less than 30) and oligoasthenoteratozoospermic (with all the three sperm abnormality). Specimens containing <10\(^6\) round cells/ml were included in the study. Sperm-free seminal plasma was obtained by centrifugation at 2500 g for 10 min and the aspirate was kept frozen at −80°C till the assay.

**Blood serum separation**

Freshly drawn non-heparinized blood was allowed to coagulate for 1 h and centrifuged at 6000 g for 10 min. The aspirated blood serum was stored at −80°C till assay.

**Determination of CAT and SOD activity**

CAT activity was estimated by the method discussed previously\(^11\). CAT degrades hydrogen peroxide, which decreases the absorbance with time and can be measured directly at 240 nm. The difference in extinction per unit time was a measure of CAT activity. The CAT activity was then calculated from the change in absorbance and expressed as U/ml.

SOD activity was measured according to the previously discussed method\(^12\). Superoxide anion radical is involved in the autoxidation of pyrogallol. At alkaline pH, SOD dismutases superoxide, thereby inhibiting the autooxidation of pyrogallol. The absorbance of each sample was measured at 420 nm before the addition of pyrogallol. The increase in the absorbance was measured at 30 s interval up to 3 min. The SOD activity was expressed as U/ml.

**Glutathione assay**

Reduced glutathione was assayed as described elsewhere\(^13\). At alkaline pH, 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacts with thiol group to produce a yellow colored compound p-nitrophenol anion, which was quantified colorimetrically.

**Malondialdehyde (MDA) assay**

MDA (a marker of lipid peroxidation) assay in blood serum and seminal plasma was done by TBARS...
Briefly, 1 part of each sample was added with 2 parts of TBA reagent (15% v/v trichloroacetic acid and 0.25 N HCl). The mixture was treated in a boiling waterbath for 13 min. TBA formed coloured adducts which were quantified using spectrophotometry analyses. After cooling, it was centrifuged at 4000 g for 10 min and supernatant was removed and absorbance was measured at 532 nm.

**Statistical Analysis**

Differences between groups were assessed using Mann-Whitney U test. Coefficients of correlation were calculated using Spearman correlation analysis. All hypothesis tests were two-tailed with statistical significance assessed at the p value <0.05 level with 95% confidence intervals. The data were expressed as the mean ± SEM. Statistical computations were calculated using SPSS 11.5 for windows software.

**Results and Discussion**

The healthy normozoospermic controls had mean sperm count >20 million per ml, progressive motility (sum of grade a and b motile sperms) >50 and normal morphology was > 30%, which was in accordance with WHO guidelines for healthy fertile men (Table 1). Significant differences were observed between the antioxidants/MDA levels in blood of patients and controls. The relationship observed between the sperm parameters and antioxidants/MDA in blood and seminal plasma of cases and controls is given in Table 2. Spearman coefficient (p), if less than 0.05 indicated significant relationship between the two variables.

The study confirmed the presence of SOD, GSH and CAT, the antioxidants considered to be potentially protective to spermatozoa and MDA, a major product of lipid peroxidation (LPO) in both blood and seminal plasma. Association of antioxidants and MDA in blood and seminal plasma with sperm parameters suggested either interaction of antioxidant molecules in blood and seminal plasma or the induction of OS in somatic cells with increase in free radical concentration in reproductive tract or germ cells.

Positive correlation of sperm count with blood SOD in both cases and controls indicated that SOD evaluation in blood could be an indicator for the analysis of oxidizing environment of sperms and can be a biochemical parameter to quantify the sperm concentration. Positive correlation between seminal SOD with abnormal sperm morphology, percentage of dead sperms in patients could be due to the abortive apoptosis (where the fas tagged and p53 expressed defective sperms escape the apoptotic mechanism) and poor semen parameters which are positively correlated. Since in healthy controls, the mechanism of abortive apoptosis was not executed to a very high degree, thus the controls did not have increased number of dead sperms or sperms with abnormal morphology.

SOD accelerates the rate of $O_2^-$ decay to at least $10^9$ times faster than the spontaneous decay. Samples with lower progressive motility of spermatozoa showed decreased SOD, GSH and CAT activity, in comparison to samples with higher progressive motility index. This suggested that the SOD, GSH and CAT along with other antioxidants contributed in maintaining non-oxidizing environment around and within the sperm as a result of which the number of mitochondrial mutations decreased as compared to patients with higher MDA or lower antioxidant profile. OS induces damage in both mitochondrial and nuclear DNA.

Since the mtDNA has only exonic regions, even single nucleotide change can be mutagenic and may impair the energy production and have profound phenotypic effect. In earlier studies from our laboratory, we reported higher percentage of mitochondrial mutations in infertile men and in this same cohort of men found low antioxidant levels and high percentage (75-80%) of sperms with abnormal morphology and impaired

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**Table 1—Detail of sperm parameters and their subclassification in patients and fertile controls**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Concentration (10^6/ml)</th>
<th>Motility (%)</th>
<th>Abnormal morphology (%)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
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</tr>
<tr>
<td>Normozoospermic (n = 40)</td>
<td>31 ± 4.01</td>
<td>69.81 ± 1.39</td>
<td>11.62 ± 1.04</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
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<tr>
<td>Oligozoospermic (n = 15)</td>
<td>0.32 ± 1.31</td>
<td>57.81 ± 1.08</td>
<td>15.53 ± 0.39</td>
</tr>
<tr>
<td>Asthenozoospermic (n = 17)</td>
<td>3.1 ± 2.01</td>
<td>20.31 ± 1.79</td>
<td>21.07 ± 1.04</td>
</tr>
<tr>
<td>Teratozoospermic (n = 19)</td>
<td>2.7 ± 1.05</td>
<td>60.81 ± 2.09</td>
<td>80.62 ± 1.056</td>
</tr>
<tr>
<td>Oligoasthenoteratozoospermic (n = 9)</td>
<td>0.35 ± 1.67</td>
<td>30.81 ± 1.43</td>
<td>77.68 ± 1.87</td>
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The negative correlation of CAT with percentage of dead and abnormal sperms clearly suggested the importance of CAT in scavenging the hydroxyl radical, a potent free radical in the induction of OS. The negative correlation of CAT with percentage dead sperms and negative correlation with progressive motility was observed in both patients and controls. No correlation could be established between the blood MDA with sperm count and progressive motility in patients and controls. Seminal MDA did not exhibit any correlation with sperm count, percentage abnormal sperms. Although similar findings were reported by Suleiman et al.21, but our findings were in contrast to other report22.

Positive correlation between GSH levels in blood and seminal plasma with sperm count and progressive motility in patients and controls. Seminal MDA did not exhibit any correlation with sperm count, percentage abnormal sperms. Although similar findings were reported by Suleiman et al.21, but our findings were in contrast to other report22.

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Infertile men included in this study had low antioxidants in their seminal plasma and blood. Low antioxidant concentration in infertile men was associated with increase in the reactive free radicals. In the initial stages of exposure to high ROS levels the antioxidant level is also increased as a counter defence mechanism to prevent the cellular damage, but on prolonged exposure the antioxidant mechanism breaks down, leading to increased levels of ROS. These increased free radicals react with the nucleotides in the DNA to induce breaks by oxidation of bases or may lead to base modifications particularly methylation and demethylation. When these aberrations accumulate beyond the capacity of repair, it leads to fragmentation of DNA.

DNA damage in germ cells adversely affects both assisted reproduction techniques (ART) and natural conception outcome and has long life implications on health of offspring. It also leads to recurrent early pregnancy loss and 3-fold increase in miscarriages. It has been reported that children conceived through ART have higher incidences of genitourinary, musculoskeletal abnormalities and cancer. There is also 6-fold increase in epigenetic and genetic defects and these can be attributed to increased sperm DNA damage due to OS. Thus, the role of sperm goes beyond fertilization and critically affects embryogenesis.

In an earlier study, our group reported higher percentage of Yq microdeletion and mt mutations in sperm DNA as compared to genomic DNA isolated from blood. Highest number of mt mutations were detected in ND (NADH dehydrogenase) group of genes. ROS levels were significantly elevated in infertile men, as compared to controls. As highlighted in this study, blood and seminal antioxidants showed a parallel relationship, indicating that ROS produced at supraphysiological levels in germ cells of infertile men induced damage to sperm nuclear and mt DNA and probably also induced OS in somatic cells. As sperm lose majority of cytosolic enzymes during spermiogenesis, supraphysiological levels of ROS induce more damage in sperm DNA as compared to somatic DNA.

Semenal OS can be detected by chemiluminescence or can also be predicted by presence of sperms with retention of cytoplasmic droplet, impaired motility, and increased viscosity of semen. Various factors can help in lowering OS like treatment of testicular or systemic infection/inflammation, exercise in moderation, stopping intake of alcohol, smoking, and a diet rich in fruits and vegetables (antioxidants).

In conclusion, the study demonstrated that blood SOD, GSH and CAT showed a parallel relationship with sperm count. A positive correlation between the blood MDA with percentage of abnormal morphology and dead sperms was also observed. Thus, based on the results, it might be possible to use these antioxidants in blood as biochemical markers to quantitate the sperm parameters. However, further studies with larger sample size are required to confirm these findings. The findings suggest that evaluation of oxidative status in both seminal plasma and blood serum is of diagnostic importance in the evaluation of male infertility and is of more clinical and prognostic value as compared to standard semen parameters.

References
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<tbody>
<tr>
<td>19</td>
<td>Halliwell B &amp; Cross C E (1994) <em>Environ Hlth Perspect</em> (Suppl 10) 102, 5-12</td>
</tr>
<tr>
<td>23</td>
<td>Li T-K (1975) <em>Biol Reprod</em> 12, 641–646</td>
</tr>
</tbody>
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