Oxidative stress and sperm mitochondrial DNA mutation in idiopathic oligoasthenozoospermic men

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Physiological function of reactive oxygen species (ROS) has been known since a long, but recently toxic effects of ROS on spermatozoa have gained much importance in male infertility. Mitochondrial DNA (mtDNA) is believed to be both source and target of ROS. mtDNA unlike nuclear DNA is not compactly packed and hence more susceptible to oxidative stress (OS) than nuclear DNA. In the present study, the role of OS in mitochondrial genome changes was studied in men with idiopathic infertility. The study included 33 infertile oligo-asthenozoospermic (OA) men and 30 fertile controls. Semen analyses were performed and OS was measured by estimating the level of malondialdehyde (MDA) in the seminal plasma and ROS in the sperm. Sperm mtDNA was sequenced by standard PCR-DNA sequencing protocol for ATPase and nicotinamide adenine dinucleotide dehydrogenase (ND) groups of genes. Sperm count and progressive motility were found to be significantly lower in infertile group than the fertile controls. Semen MDA and ROS levels of infertile group were significantly higher (p<0.0001), when compared to the control group. However, catalase and glutathione peroxidase (GPx) levels were significantly lower in infertile group, compared to controls, but no significant difference in superoxide dismutase (SOD) activity was observed between control and cases. This might be due to higher expression of SOD alone in order to overcome OS in the semen. mtDNA analysis showed significant and high frequency of nucleotide changes in the ATPase (6 and 8), ND (2, 3, 4 and 5) genes of infertile cases compared to the controls. Hence excess ROS and low antioxidant levels in the semen might cause mtDNA mutations and vice versa in OA men that might impair the fertilizing capacity of spermatozoa. Thus, it is important to understand the etiology of mitochondrial genome mutations in idiopathic OA cases for better diagnostic and prognostic value in infertility treatment/assisted reproductive technique.

Keywords: Reactive oxygen species, Oxidative stress, Infertility, Antioxidant enzymes, mtDNA mutations, Assisted reproductive technique, Sperm, Oligoasthenozoospermic

Oxidative stress (OS) is a condition established due to an imbalance between antioxidant levels and reactive oxygen species (ROS). It has been reported to be a causative factor in a variety of diseases like diabetes, cancer, aging, Alzheimer, atherosclerosis, rheumatoid arthritis, multiple sclerosis, muscular dystrophy, cancer, etc, but recently, their role on male infertility has been emphasized. Cytogenetic abnormalities, Y chromosome deletion and high testicular temperature are important aetiological factors in idiopathic cases; however, role of ROS is still unclear in male infertility. The smoking, exposure to high temperature, pollution, toxic chemicals, endocrine disruptors and genitourinary infections have also been reported to be indirectly associated with OS.

ROS could damage different parts of the spermatozoa, including both nuclear and mitochondrial (mt) DNA, but unlike somatic cells, mature spermatozoa lack cytoplasm. Since cytoplasm is the major source of anti-oxidants, lack of cytoplasm in the mature spermatozoa causes deficiency in both antioxidant defense and endogenous repair mechanism. However, naturally the deficiency of antioxidant system is compensated by the enzymatic and non-enzymatic antioxidants in seminal fluid. So, decreased antioxidant enzymes or increased ROS level disrupts the physiological function of spermatozoa and impairs sperm motility and the process of fertilization, though low ROS levels facilitate capacitation, acrosome reaction and hyperactivation.

The unique structure of sperm is well-defined with compact packing of the nuclear DNA that passes genetic information to the offspring. However, the
plasma membrane of spermatozoa possesses high amount of polyunsaturated fatty acids (PUFAs), which are highly susceptible to ROS\textsuperscript{10}. The excess ROS production can cause damage to the normal spermatozoa by inducing lipid peroxidation, resulting in alteration of sperm function and fertilizing capacity\textsuperscript{11}. Excess ROS are known to cause sperm nuclear DNA damage\textsuperscript{12} and a high degree of sperm mitochondrial mutation\textsuperscript{13}. mtDNA is highly prone to mutation due to high turnover rate, very basic repair and proof reading mechanism and lack of histone protection. Since mtDNA are very few in numbers (100 to 1000) as compared to other cells (1 to 10 lakh in ova) and somatic cells (10\textsuperscript{2} to 10\textsuperscript{4} copies), mtDNA mutations in sperm manifest early as hypospermatogenesis and later as motility defects.

As sperm motility is most important indicator of fertility potential, mt mutations have profound adverse effects on sperm function. Thus, it is important to distinguish and characterize the cause of impaired sperm function, whether it is in nuclear genome (cytogenetic abnormality or Y chromosome deletion) or a mtDNA defect\textsuperscript{14,15}. The later defect is a good diagnostic and prognostic marker in men opting for assisted reproductive technique (ART). But, it is important to understand the aetiology of mt mutations in infertile men. Thus, in this study, attempts have been made to assess OS and correlate it with mtDNA mutation in infertile men with oligozoospermia and severely impaired sperm motility. Though studies\textsuperscript{9,16} have reported OS and mtDNA mutation independently, to our knowledge, this is for the first time, when OS and mtDNA mutations have been reported in same set of infertile patients.

Materials and Methods

Subjects

The study included 33 infertile patients with idiopathic infertility and 30 proven fertile controls. The average age of infertile and control group was 30.5 and 31 years respectively. Men with known factors of infertility such as varicocele, cryptorchidism, endocrine disorders etc., azoospermic, leukocytospermic and highly viscous semen samples were excluded from the study. Those who fathered in last two years were considered as fertile controls. The study was conducted in the Laboratory of Molecular Reproduction and Genetics, Department of Anatomy, AIIMS. The subjects were referred from Department of Urology, AIIMS and Army Research and Referral Hospital, New Delhi. Informed written consent was obtained from the participant and the study was approved by the Ethical Clearance Committee of AIIMS before enrolment of the subjects.

Semen evaluation

Semen sample was collected in a sterile plastic container after sexual abstinence of 3-5 days. Semen was allowed to liquefy at room temperature for 30 min and semen parameters such as sperm count, progressive sperm motility grade and sperm morphology were evaluated according the WHO criteria\textsuperscript{17}. After complete evaluation, the semen was centrifuged at 300 g for 7 min and aliquots were prepared and stored at -80°C for the estimation of sperm malondialdehyde (MDA) and seminal antioxidant enzymes.

ROS and MDA estimation

Levels of ROS were measured in fresh washed sperm suspension using a chemiluminescence assay\textsuperscript{18} and the results were expressed as 10\textsuperscript{6} counted photons per min (cpm) per 20 x 10\textsuperscript{9} sperm. The lipid peroxide product MDA in the seminal plasma was measured as described previously\textsuperscript{19} and its levels were expressed as nmol/dl.

Antioxidant enzymes

The levels of superoxide dismutase (SOD)\textsuperscript{20}, catalase\textsuperscript{21}, and glutathione peroxidase (GPx)\textsuperscript{22} in the seminal plasma were estimated by standard protocol methods.

DNA isolation and sequencing

DNA from sperm was isolated according to previously described method\textsuperscript{23} and sequenced after running polymerase chain reaction (PCR). Primer sequences for the mitochondrial genes [ATPase and NADH (ND2, ND3, ND4 and ND5)] were obtained as described elsewhere\textsuperscript{24}. PCR amplification conditions\textsuperscript{16} were as follows: initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 2 min and final extension at 72°C for 7 min. Sequencing of PCR products was carried out using 100.0 ng (2.0 µl) of PCR product and 4 pmol (1.0 µl) of primer (forward), 4.0 µl of BigDye Terminator ready reaction kit and 3.0 µL of double-distilled water to adjust the volume to 10.0 µL. Cycle sequencing conditions were: 30 cycles at 96°C for 10 s, 50°C for 5 s, and 62°C for 4 min. Samples were dissolved in 10 µl of 50% Hi-Di formamide and
analyzed in automated DNA analyzer (Applied Biosystems). The obtained sequence was aligned using the Auto assembler and compared with the reference sequence\textsuperscript{25} to find out the nucleotide changes.

Ultrastructural sperm study

Sperm samples of OA cases were subjected for studying ultrastructural changes by transmission electron microscopy (TEM). Samples were fixed in cold Karnovsky fixative and maintained at 48°C for 2 h. Then samples were washed in 0.1 mol/L cacodylate buffer (pH 7.2) for 12 h, post-fixed in 1% buffered osmium tetroxide for 1 h at 48°C, followed by dehydration and then embedded in Epon araldite. Ultrathin sections were cut with a Leica Ultracut ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate, and then observed and photographed.

Results and Discussion

Standard semen analysis showed significantly lower sperm count, progressive motility in the infertile (OA) cases when compared to the controls. However, the levels of MDA were found to be significantly (p<0.0001) higher in infertile group (0.273 ± .03 nmol/dl) compared to the fertile controls (0.126 ± .02 nmol/dl) (Table 1). ROS level was significantly (p<0.0001) elevated in OA group compared to control men (25.06 ± 9.43 vs 0.229 ± 0.19) Catalase activity in the seminal plasma of infertile group was found to be significantly lower (p<0.001) compared to the control group. Similarly, the GPx activity was significantly lower (p<0.001) in the infertile group, compared to the fertile controls, but no significant difference was observed in SOD level between the two groups (Table 2).

mtDNA sequence analysis revealed a high frequency of nucleotide changes in the infertile cases in the genes ATPase6, ATPase8, ND2, ND3, ND4 and ND5. Significant nucleotide (nt) changes in the genes [ATPase6 (nt 9098), ATPase8 (nt 8394, 8701, 8860, 8879), ND2 (nt 4769, 5400), ND3 (10165, 10172, 10207, 10398, ND4 (nt 11719), and ND5 (nt 12705, 13707, 13708, 13946)) of infertile groups were observed compared to the control groups (Table 3). These nucleotide substitutions were significantly higher in OA cases, as compared to controls. Though maximum number of OA cases showed mutations in ATPase gene, maximum mutations/substitution were detected in ND gene.

Ultrastructural studies of sperm in OA group revealed severe disruption of microtubular normal (9 + 2) arrangement in 55% of the cases. The microtubules were disorganized and partially formed or completely absent in 43% of the patient samples and lacked dynein and nexin arms (Fig. 1). The outer fibrous sheath was also disrupted and partially formed in 62% of the OA samples. These changes were most clearly distinguishable in region of midpiece as compared to principal piece in OA group (Fig. 2), whereas only 15-22% of ultrastructural changes were observed in control samples.

OS is a well-established condition in variety of diseases, including male infertility. However, the reason for the sperm dysfunction in idiopathic infertility is still under debate. When semen analysis fails to detect the underlying pathology of sperm dysfunction, molecular analysis like cytogenetics and Y chromosome screening have been considered as important tools for couples opting for ART\textsuperscript{3,5,14,15,26}.

Generally, antioxidants are present in cell to scavenge the free radicals generated in the environment and thus prevent the damage caused to the cell. But, in spermatozoa, due to shedding of cytoplasm during spermiogenesis, the absence of enzymatic antioxidants is usually compensated by the seminal constituents of different origins\textsuperscript{27}. In our study, the antioxidants enzymes (catalase and Gpx) were found to be significantly lower in cases when

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**Table 1—Seminal parameters of infertile and control groups** [Values in parentheses are expressed as median (minimum, maximum range)]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sperm count (x 10^6/ml) ± SD</th>
<th>Progressive motility (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile (n = 33)</td>
<td>13.80 (2.5, 19.5)*</td>
<td>12.6 (3.50, 25.6)*</td>
</tr>
<tr>
<td>Controls (n = 30)</td>
<td>56.20 (35.6, 91.60)</td>
<td>67.10 (44.6, 88.4)</td>
</tr>
</tbody>
</table>

* p<0.0001 considered as significant by MannWhitney test

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**Table 2—Seminal antioxidant enzymes of infertile and control groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase (U/L)</th>
<th>GPx (U/mg protein)</th>
<th>SOD (U/ml)</th>
<th>MDA (nmol/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile (n = 33)</td>
<td>239.0± 11.96*</td>
<td>120.6 ± 8.9*</td>
<td>2.87 ± 0.28NS</td>
<td>0.273 ± 0.03*</td>
</tr>
<tr>
<td>Controls (n = 30)</td>
<td>307.5 ± 15.29</td>
<td>166.3 ± 9.7*</td>
<td>3.0 ± 0.23</td>
<td>0.126 ± 0.02</td>
</tr>
</tbody>
</table>

*p<0.0001 considered as significant by students ‘t’ test

NS, non-significant (p = 0.192)
compared to the controls. But, there was no significant difference in the SOD levels between two groups. A similar result was reported earlier. This might be due to overexpression of SOD as a result of OS to compensate the decreased level.

MDA levels were significantly higher in the infertile (OA) group compared to controls. In OA group, elevated MDA levels, which might be due excess ROS production could decrease membrane fluidity and thus impair linear progressive motility. Hence an increased ROS and MDA levels with antioxidant status of the semen indicated that these infertile OA men were under OS. Earlier, elevated MDA levels in infertile patients and decreased antioxidant enzymes level and total antioxidant capacity in the seminal plasma of infertile patients were also reported. Decreased sperm function might be due to low antioxidant level or increased lipid peroxidation due to OS. However, decreased antioxidant enzymes and increased ROS level damage various part of spermatozoa including nuclear and mtDNA. The high levels of mutant mtDNA have been found to be strongly correlate with low sperm motility.

Table 3— Nucleotide changes in control and infertile groups

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotide position and base change</th>
<th>Infertile(^a) (OA) (n = 33)</th>
<th>Control(^b) (n = 30)</th>
<th>P</th>
<th>Odds ratio (OR)</th>
<th>95% Confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase 6</td>
<td>T9098C</td>
<td>6</td>
<td>0</td>
<td>0.0250</td>
<td>14.41</td>
<td>0.775-268.13</td>
</tr>
<tr>
<td>ATPase 8</td>
<td>C8394T*</td>
<td>16</td>
<td>1</td>
<td>&lt;0.0001</td>
<td>27.29</td>
<td>3.31-224.60</td>
</tr>
<tr>
<td></td>
<td>A8701G</td>
<td>16</td>
<td>26</td>
<td>0.0015</td>
<td>1.144</td>
<td>0.041-0.50</td>
</tr>
<tr>
<td></td>
<td>A8860G</td>
<td>31</td>
<td>14</td>
<td>&lt;0.0001</td>
<td>17.700</td>
<td>3.576-87.75</td>
</tr>
<tr>
<td></td>
<td>G8879A*</td>
<td>16</td>
<td>2</td>
<td>0.0002</td>
<td>13.17</td>
<td>2.689-64.556</td>
</tr>
<tr>
<td>ND 2</td>
<td>A4769G</td>
<td>4</td>
<td>23</td>
<td>&lt;0.0001</td>
<td>0.041</td>
<td>0.010-0.16</td>
</tr>
<tr>
<td></td>
<td>G4000C*</td>
<td>0</td>
<td>21</td>
<td>&lt;0.0001</td>
<td>0065</td>
<td>0.00-0.11</td>
</tr>
<tr>
<td>ND 3</td>
<td>C10165T</td>
<td>16</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>57.51</td>
<td>3.24-1019.4</td>
</tr>
<tr>
<td></td>
<td>G10172A</td>
<td>11</td>
<td>9</td>
<td>0.793</td>
<td>1.160</td>
<td>0.402-3.38</td>
</tr>
<tr>
<td></td>
<td>C10207T*</td>
<td>21</td>
<td>2</td>
<td>&lt;0.0001</td>
<td>26.72</td>
<td>5.34-133.68</td>
</tr>
<tr>
<td></td>
<td>G10398A</td>
<td>25</td>
<td>24</td>
<td>0.767</td>
<td>0.781</td>
<td>0.23-2.50</td>
</tr>
<tr>
<td>ND 4</td>
<td>G11719A</td>
<td>29</td>
<td>15</td>
<td>0.0020</td>
<td>7.250</td>
<td>2.042-25.744</td>
</tr>
<tr>
<td>ND 5</td>
<td>C12705T</td>
<td>28</td>
<td>14</td>
<td>0.0028</td>
<td>6.400</td>
<td>1.943-21.07</td>
</tr>
<tr>
<td></td>
<td>G13707A</td>
<td>9</td>
<td>0</td>
<td>0.0022</td>
<td>23.65</td>
<td>1.310-427.22</td>
</tr>
<tr>
<td></td>
<td>G13708A</td>
<td>16</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>57.51</td>
<td>3.245-1019.40</td>
</tr>
<tr>
<td></td>
<td>T13946A*</td>
<td>12</td>
<td>0</td>
<td>0.0002</td>
<td>35.46</td>
<td>1.989-632.29</td>
</tr>
</tbody>
</table>

P value, odds ratio (OR) & 95% confidence interval (CI) were done by Fisher’s exact test

\(^a\&b\) Row indicates number of subjects with nucleotide changes for the corresponding nucleotide position of OA and control groups, respectively

Fig. 1—Altered sperm ultrastructure in OA men showing, partially formed axoneme (pAx) and missing radial links (mRL)

Fig. 2—Sperm middle piece of OA men showing swollen mitochondria (sM) with degenerated cristae

MDA levels in infertile patients and decreased antioxidant enzymes level and total antioxidant capacity in the seminal plasma of infertile patients were also reported. Decreased sperm function might be due to low antioxidant level or increased lipid peroxidation due to OS. However, decreased antioxidant enzymes and increased ROS level damage various part of spermatozoa including nuclear and mtDNA. The high levels of mutant mtDNA have been found to be strongly correlate with low sperm motility.
A very high population of sperm with decreased motility in our cases could be due to additive effect of membrane peroxidation and mt mutation-induced decreased ATP production. However, it has also been reported that the activity of oxidative phosphorylation (OXPHOS) depends on the genetic background of mitochondria that affects the sperm motility. The sequence of the sperm mtDNA from the cases showed various nucleotide changes as shown in the Table 3. In fertile controls, at mt nucleotide position 4769, the change was A>T, replacing amino acid methionine>methionine (silent mutation), which was a synonymous change, resulting no change in the protein structure. However, at 5400 nucleotide position of ND2 gene, the change of nucleotide base was G>C, replacing valine with leucine (Valine>Leucine) which was a non-synonymous change nucleotide in control population. This novel polymorphism at 5400 (ND2) nucleotide position of mt genome might be population-specific. Since demographic Indian population is remarkably different, this change needs to be studied in detail.

As mtDNA are source of production of ATP through OXPHOS, they are also site of ROS production and first site of ROS-induced damage. Moreover, increased MDA may damage or alter DNA through oxidation or covalent binding. OS may also damage or alter the DNA sequence of mitochondrial genome, thus decreasing the production of ATP and further increasing the leakage of free radicals through electron transport chain. As during spermatogenesis, there is an increase in mtDNA copy per cell, defect in mitochondrial genome may increase the free radical production in the sperms, as compared to other somatic cells. Thus, increased ROS levels and the OS status in OA men might have disturbed the ultrastructure formation in the sperm during developmental stage or at post-spermatogenesis. Moreover, sperms with mtDNA mutation may also have high nuclear DNA fragmentation, which is a pro-mutagenic change that could generate mutation in the offspring. Thus, screening of mtDNA is necessary to understand the etiology of motility disorders in OA men. Such OA cases which harbor OS-induced mtDNA mutations have a better diagnostic and prognostic value in ART, as there is no paternal transmission of mtDNA.

In conclusion, OS and mtDNA mutations play an important role in the impaired fertility of OA men. Therefore, it is necessary to understand the etiology for the impaired sperm parameters of OA men for better management of infertility treatment/ART.

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