Necessity of nuclear and mitochondrial genome analysis prior to assisted reproductive techniques/intracytoplasmic sperm injection

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Assisted reproductive technique (ART) has revolutionized the management of severe male factor infertility and in some countries 5% babies are conceived through ART/intra cytoplasmic sperm injection (ICSI). However, the carry-home live birth rate after several ART cycles is low (18-25%) and this is financially, physically and emotionally crippling for the couples. Genetic factors could lead to pre or post-implantation failure and thus explain for low ART success rate. Thus, this study was planned to understand, if infertile men harbour genetic abnormalities which may be iatrogenically transmitted by ART and adversely affect growth potential of embryo. Ninety infertile men underwent semen, cytogenetic, Yq microdeletion and mitochondrial mutation analysis. Of these, 14.4% cases harboured cytogenetic abnormality, and 8.89% Yq microdeletions. A high frequency of mitochondrial mutations was found in 23 men with asthenospermia. It is important to understand that through ART genetic abnormalities are transmitted to offspring, resulting in impaired growth and development potential of embryo and poor take-home live birth rate. Thus, genetic analysis is strongly recommend in all men with idiopathic infertility who opt for ART to counsel couples and provide them with most adapted therapeutics.

**Keywords**: Genetic, Azoospermia factor, Mitochondrial DNA, Oxidative phosphorylation, Assisted reproductive techniques, Mutations

Inspite of improvements in *in vitro* fertilization (IVF) techniques and professional expertise the carry home live birth rate after several IVF cycles is less than 25%. One of the major causes of poor assisted reproductive techniques (ART) outcome is genetic abnormalities harboured in the gametes. These genetic abnormalities may be transmitted to the embryo, following ART. Such embryos/blastocysts have impaired developmental potential and thus undergo pre or post implantation failure or early pregnancy loss. So, it is very important to obtain genetic information from couple to understand the mechanism of early embryo wastage. Recurrent miscarriages in ART/IVF/ intra cytoplasmic sperm injection (ICSI) are known to be associated with parental chromosomal abnormalities, particularly reciprocal and robertsonian translocations.

Majority of couples that opt for ART suffer from severe male factor infertility. ICSI has proven to be a boon for such couples as it bypasses several steps prior to fertilization. Recent studies have reported that 30 to 40% men in reproductive age group have a qualitative or quantitative defect in sperm production. Sperms of infertile men have a higher rate of aneuploidy and structural chromosomal abnormality than those of fertile men. In addition, these men may harbour submicroscopic deletions on long arm of Y chromosome (AZF loci) in region critical for germ cell development and differentiation\(^2-5\). A large number of infertile men harbour mutation in mitochondrial DNA (mt. DNA) in genes regulating ATP synthesis via oxidative phosphorylation (OXPHOS) pathway. Defect in ATP synthesis results in decreased ATP production, leading to low levels of spermatogenesis (hypospermatogenesis) and production of sperms with abnormal morphology and impaired motility\(^6-8\). Few recent studies have reported persistence of the paternal mitochondria DNA in embryos conceived by IVF/ICSI\(^9,10\). Thus, knowledge of integrity of mitochondrial genome is also of critical

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Abbreviations: ART, assisted reproductive techniques; AZF, azoospermia factor; ICSI, intra cytoplasmic sperm injection; IVF, *in-vitro* fertilization; OXPHOS, oxidative phosphorylation.
value in infertile men opting for ART. It is important to distinguish cases with nuclear or mitochondrial mutations. Men with mitochondrial mutations manifest as oligoasthenoteratozoospermia but this can be circumvented by ART and as normally paternal mt. DNA is not transmitted to offspring, such cases thus carry a better prognosis on ART as compared to cases with nuclear genome abnormalities.

The use of sub-optimal spermatozoa in ICSI may be associated with a higher incidence of genetic abnormalities, with life long implications on health. Such ART conceived babies have higher incidence of genetic abnormalities, major and minor congenital malformations, musculoskeletal, imprinting defects and developmental delay.

Thus, it is important to understand if men with idiopathic infertility harbour genetic abnormalities. This is not only of diagnostic importance, but has prognostic significance, if such men opt for ART. Infertile men have higher ROS levels as compared to general population and sperms are exposed to oxidative stress as majority of antioxidants enzymes are shed during spermiogenesis. Oxidative stress due to high ROS levels leads to mutations in mitochondrial and nuclear genome. Thus, though optimal ROS levels play an important physiological role in capacitation and acrosome reaction. High ROS levels induce oxidative stress. Majority of mitochondrial genes have translocated to the nucleus during course of evolution to prevent ROS induced damage. As ROS are by-products of OXPHOS pathway, mt. DNA is first site of ROS-induced damage. Thus, nature has developed a mechanism to remove paternal mt. DNA once fertilization has occurred.

Keeping all these factors in consideration, this study attempts to understand the chromosomal, nuclear and mitochondrial genome abnormalities in men with idiopathic infertility, opting for ART/ICSI. Patients

A total of 90 men (mean age 29 ± 1.5 yrs) with idiopathic infertility and 100 age-matched controls were recruited for the study. These cases were enrolled only after informed consent and ethical clearance from Institute Ethical Clearance Board. The patients were referred from infertility clinics of Urology, Endocrinology and Gynecology Departments of AIIMS and other hospitals in N. Delhi. A detailed family history was recorded in a pre-designed proforma. Results of hormonal parameters were collected in all cases. Testicular cytopathology were recorded, wherever possible. Semen and cytogenetic analysis were done in all the cases. All cases were analyzed for mutations in azoospermia gene complex (AZF) on Yq and ATPase 6, ATPase 8 and cytochrome oxidase (COII) on mitochondrial genome. All cases with non-obstructive azoospermia (NOAZ) and severe oligospermia (germ count <5 millions/ml) were included in this study. Obstructive cases of azoospermia and case with excretory defect or known causes of infertility were excluded from this study.

Semen analysis

Samples were collected after minimum of 3 days and not longer than 7 days of sexual abstinence. They were obtained by masturbation and ejaculated into a clean, wide-mouthed glass or plastic container. During microscopic examination of the samples, estimates of motility and concentration of spermatozoa were performed and the presence of cells other than spermatozoa and agglutination of spermatozoa were determined. The observation for the structure of head, mid piece and tail of the spermatozoa were evaluated under oil immersion in 100X objective. A minimum of 200 sperms were screened for morphological abnormalities of head midpiece and tail.

Chromosome preparation and Yq microdeletion screening

In patients with idiopathic oligozoospermia and azoospermia, chromosome analysis was done to identify any numerical or structural chromosomal aberrations. For this, lymphocyte cultures were setup and chromosomes were analyzed by G banding. Metaphases were analyzed using cytovision software (ZEISS microscope) classified according to ISCN. Atleast 50 metaphases were analyzed and karyotyped in each case. DNA was extracted from peripheral blood and polymerase chain reaction (PCR) for Y chromosome microdeletion analysis have been done. Mitochondrial genome mutations have been screened by the direct DNA sequencing. This aids in providing comprehensive counseling to such men/couples and also in determining the future line of management in such cases.

Material and Methods

Patients

A total of 90 men (mean age 29 ± 1.5 yrs) with idiopathic infertility and 100 age-matched controls
Table 1—List of azoospermia gene complex (AZF) region primers used in this study

<table>
<thead>
<tr>
<th>STS</th>
<th>Left primer</th>
<th>Right primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sY84</td>
<td>AGAAGGGTCTGAAAGCAGGT</td>
<td>GCCTACTACCTGGACTT</td>
<td>326</td>
</tr>
<tr>
<td>sY86</td>
<td>GTGACACACAGACTATGCTTC</td>
<td>ACACACAGAGGGACAACCCT</td>
<td>320</td>
</tr>
<tr>
<td>sY87</td>
<td>TCTGCTCACCCTGGGAGGGG</td>
<td>CGCAGGCAGTAATAAGGGGA</td>
<td>252</td>
</tr>
<tr>
<td>sY127</td>
<td>TCTGCTCACCCTGGGAGGGG</td>
<td>CGCAGGCAGTAATAAGGGGA</td>
<td>274</td>
</tr>
<tr>
<td>sY134</td>
<td>TCTGCTCACCCTGGGAGGGG</td>
<td>CGCAGGCAGTAATAAGGGGA</td>
<td>177</td>
</tr>
<tr>
<td>sY254</td>
<td>GTGACACACAGACTATGCTTC</td>
<td>ACACACAGAGGGACAACCCT</td>
<td>177</td>
</tr>
<tr>
<td>sY255</td>
<td>GTGACACACAGACTATGCTTC</td>
<td>ACACACAGAGGGACAACCCT</td>
<td>126</td>
</tr>
<tr>
<td>sY14</td>
<td>GAATATTCCCGCTCCTCCGGA</td>
<td>GCTGGTGCTCCATTCTTGAG</td>
<td>472</td>
</tr>
</tbody>
</table>

STS-PCR approach. The primers used in the study were: sY84, sY86, sY87, sY127, sY134, sY255, sY254. SRY gene served as internal control (sY14) (Table 1).

Mitochondrial gene amplification and sequencing

mtDNA genes such as cytochrome oxidase II (COII), adenosine triphosphate synthase 6 (ATPase 6), and 8 (ATPase 8) from the blood cells of the men with oligosperma as well as 50 fertile men were amplified and sequenced. Primer sequences for the above genes were obtained elsewhere17. PCR was carried out in a 0.2 mL thin-wall tube using 50.0 ng of DNA, 10 pmol of each primer, 200 µM of dNTPs, 1x PCR buffer containing, and 3 U of AmpliTaqGold (Bangalore Genie). The amplification conditions used for the above genes were as follows: 95°C for 10 min, 35 cycles at 95°C for 1 min, 62°C for 1 min, and 72°C for 2 min. Amplified products were quantified by 2% agarose gel electrophoresis. PCR products were purified by treating with exonuclease I and alkaline phosphatase (New England Biolabs, Canada) at 37°C and 80°C for 15 min each. 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 (Applied Biosystems, USA), and 3.0 µL of double-distilled water to adjust the volume to 10.0 µL. Cycle sequencing was carried out in a applied biosystems thermal cycler employing the following conditions: 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 an ABI 3700 automated DNA analyzer (Applied Biosystems). The sequences obtained were aligned with a reference sequence18 using the auto-assembler to look for mutations.

Results

Ninety infertile male cases opting for ART were enrolled in this study. The mean age of these cases and of controls was 29 ± 1.5 yr and 27 ± 2.2 yr respectively. The mean duration of marriage and cohabitation was 4.2 yr. Each case underwent a detailed clinical and andrological examination to rule out any cause of infertility and only idiopathic cases were included in the study. On repeated semen analysis, 41 men were found to be azoospermic (no sperm in ejaculate) and 49 men had oligospermia (sperm count<20 millions/ml) of which six men also had teratozoospermia (abnormal morphology >70%) and 23 men had impaired motility (asthenozoospermia).

The incidence of constitutional chromosomal aberrations among ninety infertile males was 14.44% (13 out of 90). The most common chromosomal abnormality among men (4 out of 13) was numerical sex chromosomal abnormality (47, XXY) or Klinefelter’s syndrome and its variants — 47, XXY/47, XYY or mosaic of these two cell lines. Robertsonian translocation of chromosomes 13:14 and 14:15 were present in 2 cases respectively. Reciprocal translocations were present in 3 patients, whereas deletion of chromosome 6 (q23: qter) and inversion of chromosome 7 was present in single case each (Table 2). The mean follicle-stimulating hormone (FSH) levels in cytogenetically abnormal cases and normal infertile cases were 27.5 mIU/ml and 24.5 mIU/ml respectively and in controls were 4.7 mIU/ml.

On microdeletion analysis, 8.89% (8 out of 90) cases harboured Yq microdeletions in the AZF loci. None of the controls harboured deletion on long arm of Y chromosome. All the 90 cases were analyzed for the presence of absence of the 3 azoospermia factor genes (AZFa, b, c) with 7 STS markers and details of these cases are given in Table 3. Two patients had a cytogenetically detectable deletion in the Y chromosome as the size of Y chromosome was very small, as compared to normal Y chromosome at the G
Table 2—Cases with karyotypic abnormalities with details

<table>
<thead>
<tr>
<th>Case</th>
<th>Sperm count (million/ml)</th>
<th>FNAC</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Azoospermia</td>
<td>SCO I</td>
<td>47, XXY</td>
</tr>
<tr>
<td>2</td>
<td>Azoospermia</td>
<td>SCO I</td>
<td>47, XXY</td>
</tr>
<tr>
<td>3</td>
<td>Azoospermia</td>
<td>Maturation arrest</td>
<td>46, XY/47, XXY</td>
</tr>
<tr>
<td>4</td>
<td>0.03</td>
<td>Hypo spermatogenesis</td>
<td>47, XYY</td>
</tr>
<tr>
<td>5</td>
<td>Azoospermia</td>
<td>Maturation arrest</td>
<td>45, XYt (13q:14q)</td>
</tr>
<tr>
<td>6</td>
<td>2.0</td>
<td>Not done</td>
<td>45, XYt (14q:15q)</td>
</tr>
<tr>
<td>7</td>
<td>4.3</td>
<td>Not done</td>
<td>46,XY/46, XYdel(6q23-pter)</td>
</tr>
<tr>
<td>8</td>
<td>1.20</td>
<td>Not done</td>
<td>46, XY inv 7(p12-q31)</td>
</tr>
<tr>
<td>9</td>
<td>12.0</td>
<td>Not done</td>
<td>46, XY t(13:22) (q 21.2: q ter)</td>
</tr>
<tr>
<td>10</td>
<td>Azoospermia</td>
<td>Sertoli cells only</td>
<td>46, XY t (15:3)(q23:p12)</td>
</tr>
<tr>
<td>11</td>
<td>Azoospermia</td>
<td>Maturation arrest</td>
<td>46, XY t (6:1)(q23:p11)</td>
</tr>
<tr>
<td>12</td>
<td>Azoospermia</td>
<td>SCO I</td>
<td>45, X del Y (q11.23-q11.32)</td>
</tr>
<tr>
<td>13</td>
<td>Azoospermia</td>
<td>SCO I</td>
<td>45, X del Y (q11.26-q11.30)</td>
</tr>
</tbody>
</table>

Table 3—Cases details of patients with microdeletion of AZF loci

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sperm count (Millions/ml)</th>
<th>No. of cases</th>
<th>FNAC</th>
<th>Microdeletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Azoospermia</td>
<td>2</td>
<td>SCO I</td>
<td>AZF b+c</td>
</tr>
<tr>
<td>2</td>
<td>Cryptozoospermic</td>
<td>1</td>
<td>Maturation upto spermatid stage</td>
<td>AZF a</td>
</tr>
<tr>
<td>3</td>
<td>Azoospermia</td>
<td>2</td>
<td>Maturation arrest</td>
<td>AZF b</td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
<td>1</td>
<td>Not done</td>
<td>AZF b</td>
</tr>
<tr>
<td>5</td>
<td>Azoospermia</td>
<td>1</td>
<td>SCO II</td>
<td>AZF c</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>1</td>
<td>Hypospermatogenesis</td>
<td>AZF c</td>
</tr>
</tbody>
</table>

SCO I and II, sertoli cells only type I and II, respectively; FNAC, fine needle aspirated cytology

banding level. On Yq microdeletion analysis, these two patients had AZFb+c deletions. AZFa region was deleted in one case with cryptozoospermia. Upon testicular fine needle aspirated cytology (FNAC), this case had maturation till spermatid stage. AZFb region in 3 cases and an AZFe locus in 2 cases respectively were deleted. One of the two cases with AZFb+c deletion showed SCO type I on testicular FNAC. These two AZF b+c deleted cases were azoospermic. AZFb region was deleted in third case which was oligospermic. Both azoospermic cases with AZFb deletion had maturation arrest. FNAC details were not available in other cases.

Mitochondrial mutation analysis was done in 23 asthenozoospermic cases. These cases were screened for the deletions in the ATPase 6, ATPase 8 and COII gene. DNA sequences of the above genes revealed a total of 22 nucleotide substitutions in the blood cells. Of the 22 substitutions, 9 were in ATPase 6, 6 were in ATPase 8 and 7 were in COII. Of the 22 nucleotide mutations, 5 were missense and 17 were silent mutations. Mitochondrial genome mutations analysis in controls showed 12 nucleotide changes in the genome which were mostly synonymous changes.

Discussion

Genetic factors results in partial or complete disruption of testicular function2-4. As semen quality declines, there is an increase in incidence of genetic abnormalities2,19. Knowledge of these genetic abnormalities is important to prevent unnecessary empirical treatment and to determine the future management in such couples. Hence, a detailed and complete nuclear and mitochondrial genetic study is necessary in diagnostic evaluation of male infertility. This also has prognostic significance as mutant paternal mt. DNA are not transmitted in contrast to nuclear mutations and cytogenetic abnormalities may be transmitted and may result in impaired cleavage, gastrulation and has life long implications on health2,3.
Thus, there is need for complete analysis of genome by cytogenetic analysis and mutation analysis of AZF region and mitochondrial genome. This paper reports genetic aetiology in infertile men with idiopathic infertility prior to ART/ICSI treatment. Previous studies have shown an increasing frequency of chromosomal abnormalities in couples with reproductive failure\(^3,\,4,\,5,\,19\).

The incidence of constitutional chromosomal aberrations among ninety infertile males was 14.44%. In earlier study\(^20\), 12% abnormalities were reported in males undergoing ICSI treatment. The incidence of genetic abnormality increases with decline in semen quality\(^21\). About 5-10% oligospermic and 15-20% azoospermic men harbour genetic abnormality\(^2,\,3,\,5\) and knowledge of these genetic aberrations is critical in all couples opting for ART\(^22,\,23\).

We found three translocation carriers (3.33%) which is higher compared to the general population (0.1%)\(^22,\,23\) and to the infertile male population (1%). Studies in infertile men have demonstrated a significantly higher frequency of reciprocal translocation than in the controls\(^2,\,3\). Balanced reciprocal translocation carriers may not show any phenotypic defect, except that variable sperm counts, ranging from normal counts to a low sperm count (oligospermia) or even a total absence of sperm in the ejaculate (azoospermia). Even if sperm counts are not reduced, reciprocal translocation carriers have a higher risk of pre and post implantation losses or early fetal loss or abnormal pregnancy outcome due to the production of sperm with chromosome imbalances\(^2,\,3,\,4\).

Inversions are less common constitutional aberrations with an estimated prevalence of about 0.01-0.07% for pericentric and 0.01-0.05% for paracentric inversions in infertile male population. An association between autosomal inversion and infertility in men has been reported\(^23\).

In this study, deletions on long arm of Y chromosome have been observed in 8 out of the 90 infertile cases (8.89 %). This frequency is similar to that reported in French (9%), Danish (12%) and Italian population (8%)\(^24\). Yq microdeletions are detected in 12.2% azoospermic cases. No deletions are detected in controls, indicating that these deletions are specific for spermatogenic failure. Study of Yq microdeletions is of great importance in men who opt for ART. Presence of Yq microdeletions makes the Y chromosome unstable and prone to secondary larger deletions\(^3,\,22\). In some cases the deleted Y chromosome is so unstable that it is lost from the genome resulting in mosaic cell lines like 46, XY/45, XO. Male children conceived via ART in such cases may show genital ambiguity and thus the need to screen men with idiopathic infertility that may be cytogenetically normal but harbour Yq microdeletions.

FSH is a very reliable indicator of testicular function but its levels do not correlate with Yq microdeletion though they correlate with testicular phenotype\(^26\). The mean follicle stimulating and luteinising hormone levels were 24.4 mIU/ml and 9.9 mIU/ml in azoospermic and 16.6 mIU/ml and 7.5 mIU/ml in oligospermic respectively.

Mitochondria are important components of sperm midpiece and act as a source of energy for sperm motility and its normal growth and differentiation. mt. DNA encodes for several genes which play an important role in oxidative phosphorylation and production of ATP and is most prone to ROS-induced damage. Thus, it has genes only for OXPHOS and rest of genes have translocated to nuclear genome. Therefore, the mt. genome which has no introns has little room for error. Sperm have few mt. DNA and thus mutations in mt. DNA result in early phenotypic defects, which manifest as asthenospermia. Recent studies have reported a higher number of mt. DNA in sperms with impaired motility. This is due to clonal expansion of deleted mt. DNA. Mutations in the mt. genome have been shown to be associated with the poor semen parameters such as incomplete maturation\(^25\) and impaired sperm motility\(^27\).

Of the 49 oligospermic men, 23 cases with oligoasthenozoospermia underwent mitochondrial mutational analysis. Of the 23 oligoasthenozoospermic men, a A>G transition at nucleotide 8860 was found in 20 patients and another transition A to G at nucleotide 8701 was detected in 12 cases, where 6 were novel polymorphisms in the mt. genome present in almost 50% of the cases. The A>G transition mutation at 8860 and 8701 both lie in ATPase 6 gene. This amino acid change from threonine to alanine could result in defective protein production. The ATPase 6 protein is one of the seven proteins that make up the Fo subunit of the mitochondrial FoF1-ATPase synthase complex V of the electron transport chain. The accumulation of pathogenic mtDNAs having large scale deletions or point mutations and the resultant mitochondrial defects are associated with a wide variety of disorders and mitochondrial diseases.
such as muscular, neurodegenerative disease, diabetes and aging\textsuperscript{25}.

Mutations in the mtDNA of sperm result in production of either functioning or malfunctioning proteins, hence affecting sperm motility\textsuperscript{26}. Since ICSI facilitates the fertilization by the sperm which would have been rejected in the natural process, it is essential to access the quality of paternal genetic material. However, larger population studies of these mt DNA mutations in specific diseases such as male infertility and healthy controls subjects and general population may clarify the implication and role of these mt. DNA mutations and polymorphisms and their causative role in various diseases.

**Conclusion**

Results of this preliminary study highlight the correlation between increased frequency of the genetic abnormalities in infertile men who undergo ART/ICSI. Thus, a complete work up of infertile males should include a complete genetic (nuclear and mitochondrial genome) analysis. This has both diagnostic and prognostic significance. Presence of genetic abnormalities not only helps in diagnosis, but is of immense importance to prevent vertical transmission of these anomalies. However, long-term and larger studies are required to study effect of transmission of genetic abnormalities to offspring conceived through ART. Thus, all infertile men with idiopathic infertility need to undergo a thorough genetic analysis prior to ART treatment.

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**References**