Infertility affects about 15 per cent married couples half of which may be attributed to men with low sperm motility (asthenozoospermia), low sperm count (oligozoospermia) or abnormal sperm morphology (teratozoospermia). As mitochondria are the energy source for initiation, differentiation and function of the germ cells, mutation in mitochondrial genome can impair the formation of mature spermatozoa. Mutations in mitochondrial genome are identified in patients with fertility problems. However, mitochondria are also both the source and target of reactive oxygen species (ROS). ROS are normally generated at low levels by human spermatozoa in order to perform its physiological function. However, if the generation of these reactive free radicals overwhelm the antioxidant defense system, this can lead to oxidative stress, which is characterized by mitochondrial and nuclear genome damage. So both ROS and mtDNA mutations are considered to be the major aetiological factors in a variety of human diseases including male infertility. Identification of novel mutations in mtDNA of infertile patients with supraphysiological levels of ROS is considered to be important to gain better understanding of the aetiology of idiopathic infertility. Early detection and prompt antioxidant therapy can prevent ROS induced DNA damage. This has far reaching impact if such men opt for assisted reproductive technology (ART)/in vitro fertilization.

Key words Male infertility - mitochondrial mutation - mtDNA damage - oxidative stress - reactive oxygen species - spermatozoa

Infertility is the major concern among married couples when they fail to achieve conception after 1 year of regular unprotected intercourse. It affects approximately 15 per cent of couples, attempting pregnancy and in half of these cases infertility can be attributed to be due to male factor. Common causes of male infertility, include gene mutations, aneuploides, varicocele, radiation, chemotherapy, genital tract infections, and erectile dysfunction. Azoospermia factor (AZF) deletions and high frequency of Y chromosome microdeletion in semen than in blood are reported to be one of the major aetiology leading to male infertility. In about half of the male infertile patients, the cause is not clear and hence such cases are diagnosed with idiopathic infertility. Though semen analysis is the first diagnostic step in the male infertility, it fails to detect abnormalities in up to 20 per cent of subfertile males. The recent research in the field of male infertility is focused on the reactive oxygen species (ROS), which is suspected to
be one of the major causes of infertility at molecular level. ROS are generated both at exogenous and endogenous levels and can damage different parts of the spermatozoa including mitochondrial DNA (mtDNA) and thus impair sperm function. It is well known that mitochondrial dysfunction is responsible for a variety of disorders. Mitochondrial mutations are important aetiopathogenic factors in neurodegenerative and musculoskeletal disorders. But still, little attention has been paid to mitochondrial disorders of germinal tissues especially mitochondria of sperm. As mitochondria are the major source of ATP, they play a critical role in spermatogenesis, differentiation and optimal functioning of germ cells. However, genetic alteration of mtDNA may have serious consequences on normal spermatogenesis and fertilization. Several reports have shown that mitochondrial dysfunction could lead to partial or complete spermatogenesis arrest and be an important causative factor in male infertility, and recent research points to the mitochondria as the crucial targets of ROS and as regulator of cell death. So the present review deals with the role of ROS in the pathogenesis of mtDNA mutation and the importance of detection of ROS levels and antioxidant therapy in male infertility.

**Reactive oxygen species (ROS) and male reproductive system**

A free radical is defined as “any atom or molecule that possesses one or more unpaired electrons”. Reactive oxygen species are highly reactive oxidizing agents belonging to the class of free radicals. They are highly unstable oxidants that react with many biochemical substances like lipids, amino acids, carbohydrates, protein, and DNA. Therefore ROS are considered as a causative factor for a variety of diseases. Though the presence of free radicals in the spermatozoa was reported in 1943, their role in male reproductive physiology was reported later in 1989. Low levels of ROS are necessary for normal functions of spermatozoa like capacitation, hyperactivation, motility, acrosome reaction, oocyte fusion and fertilization. It has been reported that normal functions of spermatozoa are stimulated when they are incubated with low concentration of H2O2. Other species such as nitric oxide (NO) and superoxide anion (O2−) have also been shown to promote sperm capacitation and the acrosome reaction. Though it is well demonstrated that human spermatozoa can generate ROS for its normal function, excess production in some pathological conditions adversely affect sperm function. The mechanism behind this effect is ROS induced lipid peroxidation of sperm plasma membrane, which affects membrane fluidity and mobility. In addition ROS may also affect the sperm axoneme, inhibit mitochondrial function and affect the synthesis of DNA, RNA and proteins. Approximately 40 to 80 per cent of non selected fertile patients have high levels of seminal reactive oxygen species. Hence, uncontrolled and excessive production of ROS may be one of the major factors leading to infertility. The cause for the excess production of ROS in the semen was proposed to be seminal leukocytes as well as abnormal spermatozoa. A study has also reported the positive correlation of seminal ROS levels with age in the healthy men. Among the suspected radicals, hydrogen peroxide (H2O2) is found to be the major ROS producer in human spermatozoa. During infection and inflammation, activated leukocytes in the seminal plasma can produce significantly high amounts of ROS than non-activated leukocytes. Spermatozoa may also generate ROS by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of the sperm plasma membrane and nicotinamide adenine dinucleotide (NADH) dependent oxidoreductase at the level of mitochondria, which is both the target and source of ROS.

**Role of antioxidants in male fertility**

Antioxidants are group of defense system that comprises both enzymatic and non-enzymatic molecules. The enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase/glutathione reductase (SPx/SRD) system and catalase. The non-enzymatic antioxidants include ascorbate, urate, α-tocopherol, pyruvate, glutathione, taurine and hypotaurine. Normally, antioxidants are produced against oxidizing molecules to keep them in normal level by the mechanisms of prevention, interception and repair to perform its physiological functions. Since ROS have both physiological and pathological roles, these groups of antioxidants in the seminal plasma and spermatozoa maintain a steady state of ROS level in the semen by converting free radicals into non reacting substances. It is a well known fact that unlike somatic cells, mature spermatozoa lack cytoplasm. Since cytoplasm is the major source of antioxidants, 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 components of seminal fluid. It has been reported that...
semen, seminal plasma and spermatozoa from an infertile man has lower antioxidant capacity than seminal plasma from fertile men. In many pathological conditions, these combined antioxidant capacity of enzymatic and non enzymatic substances *i.e.*, total antioxidant capacity (TAC) was found to be decreased. Yadav *et al.* reported that the seminal total antioxidant power was decreased in leukocytospermic patients. Another study showed that the levels of TAC were also found to be lowered in varicocele, vasectomy reversal, varicocele with prostatitis and idiopathic infertile group. Some of the reports revealed that semen of infertile men had increased level of ROS rather than reduced antioxidant capacity. Though activities and levels of antioxidant systems decline with age and other male factors, the mechanism behind its elevation in male infertility is not well established. Apart from the above mentioned antioxidants, recently the role of L-carnitine and L-acetyl carnitine in scavenging the free radicals and protecting the cell membrane has gained much importance in the treatment of male infertility. Highest level of L-carnitine in the man is found in epididymal fluid. Since spermatozoa are stored and gain final maturity in epididymis hence sperms are thus protected from ROS induced damage in epididymis. Carnitine also provides energy readily to the spermatozoa for motility and maturation through β-oxidation of long chain fatty acids in mitochondria. So its deficiency may cause defective sperm function and immotility. Apart from having antioxidant property, it is also believed to have an antiapoptotic effect. Thus L-carnitine and L-acetyl carnitine have been used in the treatment of certain OAT patients. A double blind random study showed increased sperm motility after treatment with combined L-carnitine and L-acetyl carnitine in patient with lower baseline sperm parameters.

**Oxidative stress (OS): its pathogenic role in male infertility**

Oxidative stress (OS) is a condition that occurs when the production of ROS overwhelms the antioxidant defense produced against them. In male reproductive pathological conditions, the OS significantly impairs spermatogenesis and sperm function, which may lead to male infertility. It has been reported that infertile men are more likely under seminal oxidative stress than the fertile men as a measure of seminal total antioxidant capacity. Several studies have used ROS-TAC (Reactive oxygen species- Total antioxidant capacity) score as a measure of seminal oxidative stress to predict male infertility. Oxidative stress has been reported to cause abnormal denaturation of DNA into single stranded DNA and double-stranded DNA breaks, DNA base-pair oxidation, chromatin cross-linking, and chromosome microdeletion. Also many factors like drug intake, smoking, pollution, radiation etc., were reported to increase seminal oxidative stress which causes spermatozoa dysfunction leading to male infertility. Metals like cadmium and lead have also been reported to increase OS and damage vital parts like protein and antioxidants in the semen. Shen *et al.* reported DNA damage as a cause of oxidative stress which resulted in increased levels of specific forms of oxidative damage such as 8-hydroxy deoxy guanosine in sperm DNA. Moreover ROS can cause various types of gene mutations including deletion, point mutation or polymorphism of both mitochondrial and nuclear DNA of spermatozoa. One of the common oxidative byproducts of DNA, 8-hydroxy-2-deoxy guanosine (8-OHdG) has been reported as a biomarker of oxidative DNA damage in the spermatozoa and also ROS produced by spermatozoa were negatively correlated with the quality of sperm in the original semen.

Presence of xenobiotics, defect in the cellular mechanism that regulates free radical generation, spermatozoa with defective cytoplasmic extrusion, varicocele, defect in Sertoli cell that removes residual cytoplasm of spermatozoa are proposed to be the main causes for origin of oxidative stress. But the exact mechanism behind this origin is yet to be established. Oxidative stress has also been reported to decrease mitochondrial respiratory function. Endogenous OS by electron leakage of the mitochondrial respiratory chain adversely affects mitochondrial function. Thus oxidative stress not only affects the fertilization capacity of spermatozoa but also alters the mtDNA of the sperm. Increased numbers of mtDNA mutations have been reported in infertile men with increased lipid peroxidation, as increased malondialdehyde (MDA) levels were found in the same population of men.

**Mitochondrial genome and oxidative phosphorylation**

The human mtDNA is a closed circular extranuclear genome and contains 16,569 base pairs. It contains two strands, a guanine-rich heavy (H) and a cystosine-rich light (L) strand. mtDNA comprises 37 genes of which 13 encode essential components of oxidative phosphorylation (OXPHOS), 22 encode parameters.
tRNA genes, 1 for 12s and 1 for 16s tRNA genes required for mitochondrial protein synthesis. Unlike nuclear genes, introns are absent in mtDNA and all of the coding sequence is contiguous and overlapping. The only non-coding segment of mtDNA is the displacement loop (D-loop), a region of 1121bp that contains the origin of replication of the H-strand and the promoters for L and H strand transcription. The remaining mitochondrial OXPHOS proteins, the metabolic enzymes, the DNA and RNA polymerases, the ribosomal proteins and the mtDNA regulating factors are all encoded by nuclear genes. OXPHOS unit is an essential part of mitochondria that are the main source of ATP, and also the main source of ROS. OXPHOS or electron transport chain (ETC) associated with the generation of energy consists of 13 subunits, which include complex I (NADH-Q oxidoreductase), seven subunits of complex II (ND1 to ND6 and 4L), complex III (cytochrome b), 3 subunits of cytochrome oxidase (COX I, COX II and COX III) complex IV, and complex V (ATPase 6 and 8 subunits). Most of these protein subunits are encoded by mtDNA except for the complex II, which is encoded exclusively by nuclear DNA. So nuclear DNA also plays an indirect role in the function of mitochondria. ATP synthesis starts with the electron separated by the hydrogen atoms and then undergoes series of reactions to set up an electrochemical gradient which supplies energy for the synthetic process. As mitochondrial DNA is the first site of ROS induced attack, majority of mitochondrial genes have migrated to the nuclear genome during the course of evolution, and nature has selected only paternal nuclear DNA to be transmitted to offspring.

As human cells rely on ATP for growth, differentiation and several physiological functions, generation of ATP by mitochondria is the major and only source for cellular homeostasis. Glycolysis, citric acid cycle and β-oxidation biochemical pathways produce a high potential energy yielding reducing substance (NADH) that enters respiratory chain (RC). The electron transport through the RC complex from NADH to oxygen molecule results in release of free energy which is utilized for the production of ATP in OXPHOS. The importance of OXPHOS has been studied by using mitochondrial inhibitors. Specific OXPHOS complex inhibitors showed a decreased sperm motility. It has been demonstrated that certain chemical ETC inhibitors produce high ROS in the mitochondria. Moreover, increased sperm motility has also been observed with addition of complex II substrates like pyruvate and succinate. These studies have demonstrated the importance of OXPHOS in the production of ATP for the sperm motility. Since OXPHOS complexes are encoded by both mitochondrial and nuclear genome, pathogenic mutation in any or both of them may lead to dysfunctional RC complex resulting deficient ATP production. mtDNA deletion and mutation have been reported in a variety of diseases. However, any mutation in mitochondrial genome would ultimately affect the production of ATP, consequently may lead to abnormal spermatogenesis, impaired differentiation and hypospermatogenesis.

Role of mitochondria in spermatogenesis

Mammalian spermatogenesis occurs continuously with individual maturation of sperm through meiotic division of spermatocytes. However, it has not been well understood whether mitochondrial respiratory function is essential for the meiotic process. But during the phase of meiosis the mitochondria are round shaped and relatively small with the electron-dense matrix flattened to the outer part of the organelle. Some of the mitochondria may disrupt normal differentiation of mitochondria and could lead to defective spermatogenesis.
spermatozoa of fertile men, whereas a 7-fold increase in mtDNA number per cubic micrometer of cell takes place during spermatozoa maturation which demonstrates the higher ATP requirements and the importance of mtDNA for the sperm function. The orientation and the structure of mitochondrion in the midpiece of mature spermatozoa is also an important factor in the sperm motility. Shorter midpiece and less number of gyres was observed in asthenozoospermic men when compared with the fertile controls. It has been suspected that degeneration of mitochondria may also occur during epididymal passage. The position of mitochondria in the upper part of the sperm flagellum is unique. As mitochondria are the major source of the ATP that helps in the movement of the sperm, the mitochondria produce ATP that diffuses downwards to the axonemal microtubules and their dynein arms. Various defects or pathological changes in the arrangement of mitochondria are believed to affect the motility of the spermatozoa in idiopathic asthenozoospermic and oligoasthenozoospermic (OA) patients. Since there is an interaction between nuclear and mitochondrial gene, a study also proposed the DNA fragmentation and mitochondrial swelling as a more indicative parameter of impaired motility. Moreover, abnormal or complete absence of mitochondria or its functional impairment, sometimes all of the above may act as a sole reason for the sperm immotility due to the inability to produce ATP for the flagellar beat. Mitochondrial structure and DNA studies are also recently focused as paternal mtDNA inheritance could occur at low level in intracytoplasmic sperm injection (ICSI).

Mitochondrial DNA (mtDNA) mutation in male infertility

The location of mitochondria is unique in case of spermatozoa, where it is located at the site of maximum energy requirement i.e., around the midpiece of the spermatozoa. It has been well established that mitochondria make ATP by the coupling of respiration generated proton gradient with the proton-driven phosphorylation of ADP. However, on the other hand unlike nuclear DNA, mitochondrial DNA is not protected by histones and are physically associated with the inner mitochondrial membrane, where highly mutagenic oxygen radicals are generated as byproduct of OXPHOS in the respiratory chain and leakage of these free radicals from the respiratory chain makes the mitochondria as a major intracellular source of ROS. These unique features are probably the cause of the about 10 to 17 times faster accumulation of polymorphisms and mutation in mitochondrial DNA than in nuclear DNA. Also lack of an efficient repair system in mitochondria and abnormal mitochondrial metabolism may accelerate the rate of mitochondrial DNA mutation and therefore, higher amount of 8-OHdG have been reported in mitochondrial DNA than in nuclear DNA of infertile patient. Several single nucleotide polymorphisms (SNPs) in mtDNA have been reported in many of the diseases including male infertility. Studies have also reported low levels of mtDNA mutation in the semen of the infertile men. Cell division in the testis may be the reason for the accumulation of tissue specific mosaicism at higher level in spermatozoa.

In most of the mitochondrial disease, the expression of abnormal phenotype occurs only when the ratio of mutated or deleted mtDNA and wild mtDNA exceeds a critical threshold. For example >60 per cent mutant mtDNA are expected to express the phenotype in Leber’s hereditary optic neuropathy (LHON) patients. Some of the heteroplasmic associated disorders are mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke (MELAS), rhombomylolysis, dystonia, etc. The mixture of wild type mtDNA and altered mtDNA in the same mitochondrion called heteroplasm has gained much importance recently in the expression of the abnormal genotype, because most of the pathogenic mtDNA mutations are heteroplasmic but not all. Usually the phenotype is normal until the proportion of mutant mtDNA and the threshold for genotype expression exceeds. Though energy requiring organs like brain, muscle and heart are mostly affected by heteroplasy, such effect on sperm mtDNA is not well studied. One study has attempted to reverse the heteroplasmic mutation in mtDNA. More studies are needed to understand the role of heteroplasm in sperm mtDNA of infertile men, where homoplasmy mutant mtDNA has also been reported in OA infertile patients. As there are a few mtDNA in sperm as compared to somatic cell, mtDNA mutations manifest early and have more deleterious consequences.

In 1988, seven years after the complete sequence of human mtDNA was published, the first pathogenic mutations were discovered. Now, more than 100 point mutations and many rearrangements have been known to be associated with various human mitochondrial diseases. But so far only a few studies have reported mutation in mtDNA of the spermatozoa.
These mutations in spermatozoa could be pathogenic or common mtDNA variants that only affect male fertility because mtDNA is maternally inherited. But a study showed that individual cells must contain a high percentage of mutant mtDNA before they express a respiratory chain defect. As only a few mtDNA per mitochondrion in spermatozoa are present, an early expression of phenotype takes place. High levels of mutant mtDNA were also correlated with low sperm motility in males who had inherited the A3243G mtDNA mutation from their mother.

One study from India revealed a 9 misense and 27 silent mutations in the sperm mtDNA, but not in the blood cells DNA of an oligoasthenoteratozoospermic (OAT) man, who also had varicocele of the left testis. Though the patient was not fertile under the normal criteria, his wife conceived without varicocelectomy. But the diminished motility of the sperm has been suspected due to a 2-nucleotide deletion in the mitochondrial COII genes. Another recent study from India showed a novel missense mutation (C11994T) in the ND4 gene of oligoasthenozoospermic samples and it has been proposed that the wild type allele had totally replaced by the mutant allele over several generations in the maternal lineage leading to the mutant mtDNA homoplasy. Kumar et al. showed significant nucleotide changes in the mitochondrial gene (ATPase 6, ATPase 8, ND2, ND3, ND4 and ND 5) in the semen of the infertile men. In contrast, a study from Portugal reported no C11994T mutation in their OA infertile population suggesting that such mutation may be population specific. GSTM1 (glutathione S-transferase) in association with mtDNA 4977 deletion has also been reported in infertile Indian population.

During differentiation of germ cells in the testes, abnormal mitochondria were also reported in the spermatids and Sertoli cells of the patient with encephalomyopathy. This may lead to depletion in the energy production and thus affects spermatogenesis and could result in production of abnormal spermatozoa. However, another study revealed multiple ΔmtDNA in the biopsied tissue of an azoospermic or severe oligozoospermic patient. Recent ongoing study in our laboratory reported a high frequency of mitochondrial mutation in men with severe oligoasthenozoospermia (OA) and harboured no Y-chromosome microdeletion. Thus it is possible that mitochondrial mutations were involved in the pathogenesis of OA in this group of infertile men. In addition, men who harboured multiple mitochondrial mutations and large deletion had a much more severe phenotypic defect with the presence of immotile sperms. But studies failed to explain the exact causative mechanism behind these mtDNA mutations in the male germ cells.

A recent study in mito-mice carrying different proportions of Δmt DNA showed respiratory chain defect that leads to meiotic arrest during spermatogenesis. Thus respiration deficient spermatocytes could not complete meiosis, and were removed by apoptosis. Some of the spermatids showed abnormalities in middle piece and nucleus suggesting abnormal sperm formation. The study also confirmed that ΔmtDNA affected mitochondrial respiration chain function and resulted in oligozoospermia, asthenozoospermia and teratozoospermia leading to infertility in mice.

**ROS associated mtDNA mutation**

It is well established that increased production of ROS adversely affects sperm function leading to infertility. Production of ROS is significantly increased in dysfunctional mitochondria. This process may be due to ROS induced damage to the mitochondrial genome as ROS are byproduct of OXPHOS system. Mitochondrial dysfunction in such cases may be measured as mitochondrial membrane potential (MMP), which has been reported to decrease in the spermatozoa of infertile men with raised ROS level. Several studies have reported that human cells harbouring mutated mtDNA have lower respiratory function and show increased production of superoxide anions, hydroxyl radicals and H₂O₂. However, excess production of ROS further damages mtDNA, which is more susceptible than the nuclear genome. Thus during the course of evolution, majority of mitochondrial genes have been transferred to the nuclear genome and only those genes essential for OXPHOS are now located in mtDNA. Ultimately, impairment of electron transport chain results in enhanced production of ROS in mitochondria due to incomplete reduction of oxygen. Selvi Rani et al. reported for the first time, mutations in the sperm's mtDNA but not in the DNA isolated from blood. This study clearly suggests that genetic alteration in sperm mtDNA occurred during the differentiation or spermatogenesis or due to random segregation of mtDNA during cell division.
Also mtDNA is more sensitive than nuclear DNA to \( \text{H}_2\text{O}_2 \)-induced damage and protracted treatment leads to persistent mtDNA damage and loss of mitochondrial function. Moreover, ROS induced chain-propagating reaction is responsible for overall increase in the steady state level of mtDNA damage and it is also believed that ROS mediated damage to mitochondria may inactivate electron transport chain, thus altering normal mitochondrial function\(^{109}\). Persistence of mtDNA damage could be due to continuous ROS production by lipid peroxidation and/or damage at the electron transport chain (Fig.). Study by Yakes and Houten\(^{109}\) supports the sensitivity of mtDNA to ROS. Similar results were also reported in a study from an Indian population\(^{111}\).

Fig. Many physical, chemical and biological factors disturb mitochondrial respiratory chain and increase the production of reactive oxygen species beyond the level of antioxidant system. This establishes oxidative stress (OS) in the male reproductive system. mtDNA close to electron transport (ET) chain gets damaged by ROS and undergoes mutation, which results in the formation of defective ET chain. The altered ET chain further increases the production of ROS and also impairs the spermatogenesis and sperm function leading to male infertility.

A correlation was found between ROS and mitochondria in apoptosis, as high levels of ROS disrupt the inner and outer mitochondrial membrane and result in release of cytochrome C from the mitochondria\(^{104}\). Cytochrome C protein activates the caspases and induces apoptosis, which has been reported to be significantly higher in oxidative stress induced infertile men\(^{104}\). Though mitochondria are the major source of ATP, they are also the major source of intracellular ROS and mitochondrial disorders are primarily manifested in high energy demanding organs or tissues\(^{110}\). Since the number of mtDNA in sperm is far fewer than in somatic cell, these manifest as early phenotypic defect such as impaired motility and abnormal cytoskeleton of axoneme\(^{111}\). Increase
in number of mtDNA mutations may also lead to production of sperm with abnormal morphology and ultrastructural defects\textsuperscript{11}. However, a recent study from our laboratory showed raised ROS levels and sperm DNA (nuclear & mitochondria) damage in idiopathic infertile men\textsuperscript{52,112,113}.

Conclusions

Several studies suggest that raised ROS levels and mtDNA mutation play an important role in the pathogenesis of male infertility. The role of ROS, its physiological and pathological levels are yet to be established. Since spermatogenesis is a complex process involving various stages and different type of cells, mutations in mitochondrial genome, could disturb the formation of morphologically and functionally mature spermatozoa thus leading to infertility. For better understanding of the aetiology of male infertility, the correlation of pathological levels of ROS which lead to mtDNA mutation, needs to be established by larger studies. Infertile patients identified with elevated level of ROS may get benefit from the suitable antioxidant therapy. Since OS imbalance manifests as motility defects and DNA damage, early diagnosis of OS and prompt antioxidant treatment may prevent OS induced DNA damage. This has far reaching and important implication in men opting for ART. Further studies in this area are recommended to subgroup the idiopathic infertile patients with high oxidative stress to achieve high success rate in infertility treatment and ART.

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