

**INVITED REVIEW**

Causes and consequences of sperm mitochondrial dysfunction

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Abstract

Mitochondria have multiple functions, including synthesis of adenine triphosphate, production of reactive oxygen species, calcium signalling, thermogenesis and apoptosis. Mitochondria have a significant contribution in regulating the various physiological aspects of reproductive function, from spermatogenesis up to fertilisation. Mitochondrial functionality and intact mitochondrial membrane potential are a prerequisite for sperm motility, hyperactivation, capacitation, acrosin activity, acrosome reaction and DNA integrity. Optimal mitochondrial activity is therefore crucial for human sperm function and semen quality. However, the precise role of mitochondria in spermatozoa remains to be fully explored. Defects in sperm mitochondrial function severely impair the maintenance of energy production required for sperm motility and may be an underlying cause of asthenozoospermia. Sperm mtDNA is susceptible to oxidative damage and mutations that could compromise sperm function leading to infertility. Males with abnormal semen parameters have increased mtDNA copy number and reduced mtDNA integrity. This review discusses the role of mitochondria in sperm function, along with the causes and impact of its dysfunction on male fertility. Greater understanding of sperm mitochondrial function and its correlation with sperm quality could provide further insights into their contribution in the assessment of the infertile male.

KEYWORDS

male infertility, mitochondrial DNA, mitochondrial membrane potential, oxidative stress, sperm mitochondria

1 | INTRODUCTION

Mitochondria are multifunctional organelles found in most eukaryotic organisms. The mitochondrion forms a widespread network in many cells, which are dynamically regulated by a cascade of processes that are closely related albeit independent (Wu, Zhang, & Ren, 2019). The mitochondrion is referred to as the cell's powerhouse, as it supplies most of the cell's chemical energy in the form of adenine triphosphate (ATP). Besides generation of ATP, mitochondria play a key role in numerous basic and advanced cellular processes, including cellular homeostasis and apoptosis (Herst, Rowe, Carson, & Berridge, 2017). They are also essential for cellular signalling and

communication through various means, including reactive oxygen species (ROS). The mitochondrial electron transport chain (ETC) induces the production of ROS required in signalling pathways, but if produced in excess, can cause oxidative damage (St John, Bowles, & Amaral, 2007).

Although almost all of the cytoplasm is removed during spermatogenesis, mitochondria are retained in mature spermatozoa, which suggest its importance in male fertility (Zhang et al., 2019). Spermatozoa contain a limited number of mitochondria in the midpiece, which play a vital role in sperm function. The energy required for spermatozoa to carry out cellular processes necessary for successful fertilisation such as motility, hyperactivation,

capacitation and acrosome reaction is provided by the mitochondria. Mitochondrial activity is an integral aspect of human sperm functionality, both in terms of quality and in terms of fertilisation ability (Ramalho-Santos et al., 2009; Sousa et al., 2011).

The functionality of sperm mitochondria is also physiologically important during epididymal storage, as well as from ejaculation to movement within the female reproductive tract and in sperm-oocyte interactions (Amaral, Lourenco, Marques, & Ramalho-Santos, 2013). The sperm mitochondrion possesses its own genome, mitochondrial DNA (mtDNA) and specific ribosomes, which facilitate the local synthesis of proteins (St John, Facucho-Oliveira, Jiang, Kelly, & Salah, 2010). However, any events that lead to alterations in the mitochondrial genome or compromise the sperm mitochondrial functionality may potentially affect sperm function and consequently male fertility (Amaral, et al., 2013). This article discusses the role of mitochondria and its DNA in human sperm function, factors influencing sperm mitochondrial function and the consequences of its dysfunction on male fertility.

1.1 | The origin of the mitochondrion

There are two opposing theories concerning the origin of the mitochondrion. They differ on assumptions regarding the nature of the host, physiological capabilities of the mitochondrial endosymbiont and ecological interactions that led to physical association between both partners when symbiosis was initiated (Martin & Mentel, 2010). The more recent theory is supported by phylogenetic analysis of conserved ribosomal RNA (rRNA). It advocates that mitochondria originated from free-living bacteria (protomitochondria), which were acquired by an archaeal cell through endocytosis. The protomitochondrion had metabolic versatility to live with or without oxygen. The production of hydrogen by the endosymbiont was a source of energy and electrons that benefited the archaeobacterial host (Herst et al., 2017).

Alternatively, the traditional theory postulates that the host was an anaerobic eukaryote that acquired the mitochondrion actively via phagocytosis. The mitochondrial endosymbiont was possibly an obligate aerobe, and the primary benefit of the symbiosis was probably the endosymbiont's ability to detoxify oxygen for its anaerobe host (Martin & Mentel, 2010). The protomitochondria presented the early eukaryotes with the metabolic ability to utilise oxygen to fuel aerobic respiration instead, which is a more efficient way of releasing energy from organic substrates. This enabled them to thrive in new and diverse ecological niches, supporting the inception of complex multicellularity, which eventually gave rise to fungal, plant and animal cells (Herst et al., 2017).

Like the eukaryotes, mitochondria seem to have a single origin having arose only once in all of evolution. This was best evidenced from a preserved set of homologous and commonly inherited genes conserved in the mitochondrial DNA across all known eukaryotic groups. Eukaryotic evolution is assumed to have initially begun 1.45 billion years ago, which can be taken as the minimum age of mitochondria (Martin & Mentel, 2010).

1.2 | Mitochondrial structure, function and dynamics

The mitochondria are classically described as an organelle of elliptical shape, consisting of a smooth outer membrane that surrounds an inner membrane (Figure 1). These membranes are separated by an inter-membrane space. The inner mitochondrial membrane folds to form the cristae, which extend into the protein-dense matrix within the organelle (Cooper, 2000). The inner membrane can be subdivided into two distinct subdomains, the inner boundary membrane that interacts with the outer membrane, and the cristae membrane that forms the majority of the inner membrane surface (Vogel, Bornhovd, Neupert, & Reichert, 2006). Cristae junctions in the inner membrane connect individual cristae with the inner

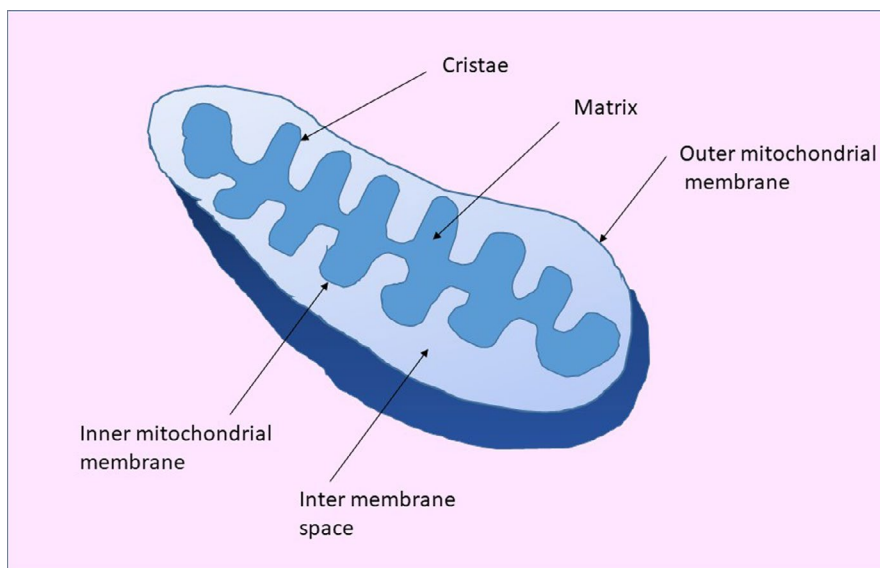


FIGURE 1 Structure of the mitochondrion

boundary membrane, forming barriers between the fluid-filled intracristal space and the inter-membrane space (Frey, Renken, & Perkins, 2002).

The mitochondria are therefore made up of three membranes, that is the outer boundary, inner boundary and cristae membranes, and three spaces, that is the inter-membrane, inter-cristal and matrix spaces (Vogel et al., 2006). Each of these structural components has very different protein compositions and distinct functional roles. The outer mitochondrial membrane is porous and contains pore-forming membrane proteins (mitochondrial porins) that permit free passage of ions and small, uncharged molecules. The most abundant protein in the outer membrane is the voltage-dependent anion channel (VDAC), which guides the metabolic flux across the outer membrane. VDAC also plays a major role in mitochondrially induced apoptosis (Bayrhuber et al., 2008).

The inner mitochondrial membrane enclosing the matrix space is a protein-rich lipid bilayer that only allows the passage of small ions and metabolic substrates using specific membrane transport proteins (Bayrhuber et al., 2008). An electrochemical membrane potential of approximately 180 mV builds up across the inner mitochondrial membrane due to its ion/molecule selectivity (Kuhlbrandt, 2015). In addition, metabolite exchange, protein assembly, translocation and degradation as well as iron-sulphur biogenesis occur in the inner membrane (Vogel et al., 2006). The cristae junctions appear to regulate the dynamic distribution of proteins, lipids and soluble metabolites between the different mitochondrial subcompartments (Zick, Rabl, & Reichert, 2009).

The main sites of mitochondrial energy conversion are the cristae. The cristae membrane contain the oxidative phosphorylation (OXPHOS) respiratory chain complexes, namely NADPH (reduced nicotinamide adenine dinucleotide phosphate dehydrogenase), succinate dehydrogenase, cytochrome bc1, and cytochrome c oxidase and ATP synthase (complexes I, II, III, IV and V respectively), as well as abundant amounts of the small soluble electron carrier protein, cytochrome c (Kuhlbrandt, 2015). Cytochrome c transports electrons from complexes III to IV, and its release into the cytoplasm can trigger apoptosis (Li et al., 1997). The high pH of the mitochondrial matrix generates a transmembrane electrochemical gradient across the inner membrane that drives ATP synthesis. Moreover, organellar DNA replication, transcription, protein biosynthesis and various enzymatic reactions occur in the mitochondrial matrix (Kuhlbrandt, 2015).

The mitochondria have three discrete functions that help in the maintenance of cellular homeostasis, namely bioenergetics, biosynthesis and signalling (Chandel, 2014). The prime function of the mitochondria is the production of ATP from ADP and phosphate ions by the mitochondrial ATP synthase during cellular respiration (citric acid cycle). As an energy-rich compound, ATP drives numerous fundamental cellular functions including protein biosynthesis and degradation, as well as generation and maintenance of membrane potentials. The mitochondria are also responsible for the production of NADH and GTP (guanosine triphosphate) in the citric acid cycle, synthesis of haem and iron-sulphur clusters, as well as phospholipids for membrane biogenesis (Kuhlbrandt, 2015).

The mitochondrion plays a key role in the generation of ROS. Superoxide anions are mainly produced in respiratory complexes I and III of the ETC (Quinlan, Perevoshchikova, Hey-Mogensen, Orr, & Brand, 2013). Mitochondrial production of ROS is controlled by the availability of oxygen to the mitochondria, redox state of the various ETC complexes and mitochondrial membrane potential (MMP) (Murphy, 2009). Mitochondria also act generally as cellular signalling hubs (Chandel, 2014) and participate in stress responses (Pellegrino & Haynes, 2015). Mitochondria play a significant role in cell survival and can facilitate apoptosis when required (Frey & Mannella, 2000). Besides apoptosis, mitochondria are also involved in ageing (Bratic & Larsson, 2013) and thermogenesis (Chouchani, Kazak, & Spiegelman, 2017). Mitochondria also transiently store calcium and contribute to cellular calcium homeostasis by acting as cytosolic buffers of calcium (Rizzuto, De Stefani, Raffaello, & Mammucari, 2012).

Homeostasis of the mitochondrion is dynamically maintained by mitochondrial fusion/fission, mitochondrial biogenesis, mitophagy and apoptosis (Huang et al., 2019). The highly dynamic mitochondria undergo coordinated cycles of fission and fusion to maintain their size, shape, number and distribution within the cytoplasm. In mitochondrial fission, one mitochondrion divides into two daughter mitochondria, whereas in fusion, two mitochondria unite to form one mitochondrion (Tilokani, Nagashima, Paupe, & Prudent, 2018). A high fusion-to-fission ratio results in elongated, tubular, inter-connected mitochondrial networks, while a low ratio leads to fragmented, discontinuous mitochondria. Both these opposing processes are carefully regulated by mitochondrial fusion proteins mitofusins 1 and 2, and optic atrophy 1 as well as fission proteins dynamin-related protein 1 and fission protein 1 (Jheng et al., 2012). This continuous network remodelling via fusion and fission mediates a quality control mechanism for the mitochondrial population (Ferree & Shirihai, 2012).

To maintain optimal functions, quality control is further mediated through regulation of mitochondrial turnover via mitochondrial biogenesis and mitophagy. Mitochondrial biogenesis involves replication of mitochondrial DNA along with synthesis and assembly of mitochondrial components to produce new mitochondria, thereby restoring mitochondrial deficits. In mitophagy, irreversibly damaged or dysfunctional mitochondria and proteins are eliminated through an autophagic process (Huang et al., 2019). During mitochondrial fission and fusion, the outer membrane must remain tightly sealed without any leakage of cytochrome c from the cristae to prevent apoptosis from being triggered (Kuhlbrandt, 2015). These kinds of plasticity confer the mitochondria with the adaptive flexibility needed to adjust to varying cellular stresses and metabolic demands (Ferree & Shirihai, 2012).

2 | THE MITOCHONDRIAL GENOME

2.1 | Mitochondrial structure and gene expression

Mitochondria possess their own genome in the mitochondrial matrix, termed mitochondrial DNA (mtDNA). The mitochondrial genome is

a closed, circular, double-stranded DNA molecule of around 16,569 base pairs. The mtDNA contains 37 genes that encode for 13 polypeptides, 22 mitochondrial transfer RNAs (mt-tRNAs) and two ribosomal RNAs (mt-rRNAs) (Taanman, 1999). The heavy strand contains most of the genes and encodes two rRNAs, 14 tRNAs and 12 polypeptides, whereas the light strand codes for eight tRNAs and one polypeptide. The 13 mtDNA-encoded polypeptides comprise the subunits of respiratory chain enzyme complexes and ATP synthase of the OXPHOS system, with seven genes coding for complex I, three for complex IV, two for complex V and one for complex III (Taanman, 1999).

The majority of the mtDNA duplexes contain a displacement loop (D-loop), where a short nucleic acid strand that is complementary to the light strand displaces the heavy strand. This D-loop region contains the leading-strand origin of replication and major promoters for transcription, thereby serving as the main regulatory site for mtDNA expression. Mitochondria are not a self-sustaining cellular organelle despite having its own genome. Several trans-acting nuclear-encoded factors support its replication and transcription (Taanman, 1999). A single-subunit RNA polymerase (POLRMT) provides the promoter-binding specificity and catalytic polymerase activity that drives mtDNA transcription (Gaspari, Falkenberg, Larsson, & Gustafsson, 2004). Additionally, other factors such as mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B2 (TFB2M) and mitochondrial transcription elongation factor (TEFM) are essential for efficient transcription (Minczuk et al., 2011). The individual mt-mRNA, mt-rRNA and mt-tRNA transcripts undergo endonucleolytic processing followed by post-transcriptional modifications (Pearce et al., 2017).

Approximately 1,500 nuclear proteins contribute to the mitochondrial proteome (Herst et al., 2017). Nuclear-encoded proteins are synthesised on cytosolic ribosomes with a cleavable, N-terminal pre-sequence for mitochondrial targeting, after which they are transported into the mitochondria (Taanman, 1999). Nucleoid mitoproteins expressed in the cytoplasm comprise the enzymes required for the citric acid cycle, biosynthesis of lipid, nucleic and amino acids, transcription factors, mtDNA and RNA polymerases, and ribosomal proteins along with the DNA repair pathway proteins (Herst et al., 2017). These nucleoid mitoproteins are folded as they pass through the outer mitochondrial membrane via the translocases of the outer/inner membrane (TOM/TIM) complex, after which they are translocated to their specific sites in the mitochondrial subcompartments (Hensen, Cansiz, Gerhold, & Spelbrink, 2014).

2.2 | Mitochondrial DNA homoplasmy and bottleneck

Each somatic cell mitochondria harbours around 5–10 copies of mtDNA, and a total of 1,000–5,000 copies per cell (Giles, Blanc, Cann, & Wallace, 1980). This polyploidic characteristic of the

mtDNA is the basis for an important feature of the mitochondrial genome, homoplasmy and heteroplasmy. In a physiological state, homoplasmy occurs when all copies of the mtDNA are identical, whereas heteroplasmy signifies the presence of a mixture of two or more mitochondrial genotypes (Taylor & Turnbull, 2005).

Human mitochondria and mtDNA are characteristically transmitted to the subsequent generation exclusively through maternal lineage (Wallace, 2007). Therefore, an individual normally has only a single (maternal) mtDNA genotype with mitochondrial genomes that are almost genetically identical (homoplasmy) (Luo et al., 2018). There are several mechanisms through which homoplasmy with normal mtDNA in mature oocytes is maintained in the fertilised egg and early embryo (Hirata, Hoshi, Shoda, & Mabuchi, 2002). These include the bottleneck mechanism in oogenesis that acts in a coordinated manner with the mechanism for elimination of abnormal mtDNA (e.g. apoptosis). Along with these mechanisms, paternal mtDNAs in the sperm midpiece are degraded in the fertilised egg, resulting in maternal inheritance of mtDNA (Hirata et al., 2002).

During oogenesis, replication of mtDNAs occurs from either a single or very few template mtDNA to help establish homoplasmy in the oocyte (Hirata et al., 2002). This mitochondrial genetic bottleneck during oocyte development affects the variability of mtDNA transmission through the maternal germline. As the genetic bottleneck in the female germline reduces the mtDNA copy number within a cell, it could lead to uneven distribution of wild-type and mutated genomes across generations. This could then result in key shifts in the levels of heteroplasmy (van den Ameele, Li, Ma, & Chinnery, 2020). If wild-type and mutant maternal alleles co-exist with the normal genotype (heteroplasmy), then this could result in mtDNA dysfunction and mitochondrial disease. The severity of these would depend on the varying extent of heteroplasmy in the tissues (Luo et al., 2018).

2.3 | Differences between mitochondrial DNA and nuclear DNA

The mitochondrial and nuclear genomes can be differentiated based on three major aspects (Taylor & Turnbull, 2005). Firstly, mitochondrial genes are exclusively maternally inherited and do not follow a Mendelian pattern of inheritance. Secondly, the mitochondrial genome normally exhibits a state of homoplasmy, but polyploidy (heteroplasmy) may co-exist within a cell. Thirdly, mtDNA species have the capability to change constantly over time or by mitotic segregation during cell division. In contrary, diploid nuclear genes normally acquire a few states such as homozygous wild-type, heterozygous or homozygous mutant (Taylor & Turnbull, 2005). Mitochondrial DNA is not packaged as nucleosomes are (as nuclear chromatin) due to the lack of histones. The mtDNA molecule, however, forms a spherical nucleoprotein complex with over 20 different proteins, termed nucleoid, which contains a copy or more of mtDNA (Lee & Han, 2017). Further comparisons between the mitochondrial and nuclear genomes are shown in Table 1.

TABLE 1 Differences between human mitochondrial and nuclear genomes

	Mitochondrial DNA	Nuclear DNA
Location	Mitochondrial matrix	Cell nucleus
Size	16,569 base pairs	~3.3 × 10 ⁹ base pairs
Structure	Double-strand, circular	Double helix, linear, packaged in chromosomes
Cell division	Fusion/fission (continuous)	Cell cycle regulated
DNA copies per cell	~10–50,000	2 (1 allele from each parent)
DNA molecules per cell	Hundreds to thousands of copies per cell	Haploid cells: 23; Diploid cells 46
Total DNA	0.25%	99.75%
Chromosomes	1 (polyploidy: 2–10 per organelle)	22 autosomes, 2 sex
Chromosomal pairing	Haploid	Diploid
Inheritance	Maternal	Mendelian inheritance for autosomes and X chromosome; Paternal inheritance for Y chromosome
Number of genes encoded	37 (13 protein coding and 24 nonprotein coding), that is 13 polypeptides, 22 tRNAs and 2 rRNAs	~20,000–30,000 protein coding
Gene density	1 per 450 base pairs	~1 per 40,000 base pairs
Introns	Absent	Present in almost every gene
Codon	Unique genetic code: AUA encodes methionine (instead of isoleucine) AUU (ATA) encodes a methionine start codon (instead of isoleucine) AGA and AGG encode a stop codon (instead of arginine) UGA (TGA) encodes tryptophan (instead of a stop codon)	Universal genetic code
Coding DNA	~93%	~3%
Noncoding sequence	Few noncoding sequences (~7%)	Many noncoding sequences (~98%)
Associated proteins	No histones Mostly protein-free Associated with few proteins (e.g. TFAM) that form nucleoids	Nucleosome-associated histone proteins Nonhistone proteins
Replication	Strand-coupled and strand-displacement models Involves three nuclear-encoded protein complexes: <ul style="list-style-type: none"> • DNA polymerase gamma • TWINKLE DNA helicase • Mitochondrial single-strand binding protein (mtSSB) 	Strand-coupled mechanism Involves DNA polymerases alpha and beta delta
Replication pair	No	Yes
Transcription	Bulk transcription for whole strands (polycistronic) POLRMT, TFAM, TFB2M, TEFM	Individual gene transcription (monocistronic)
Copy number	Hundreds to thousands per cell	One set per cell
Recombination	Occurs at a cellular level but unlikely at a population level	Occurs during the prophase of meiosis—each pair of homologues recombines
Generational recombination	No	Yes
Mutation rate	5–10× that of nuclear DNA	Low
DNA repair enzymes	Polymerase gamma	Multiple
Reference sequence description	Anderson et al. (1981)	Lander et al., (2001)

Abbreviations: rRNAs, ribosomal RNAs; TFAM, mitochondrial transcription factor A; tRNAs, transfer RNAs.

Adapted from: Strachan and Read (1999) and Taylor and Turnbull (2005).

2.4 | Susceptibility and mutations of mitochondrial DNA

Mutations in the mtDNA are associated with various types of human diseases, including male infertility (Shamsi et al., 2008; Jiang

et al., 2017; Venkatesh, Deecaraman, Kumar, Shamsi, & Dada, 2009). The mitochondrial genomes appear to have a higher load of deleterious mutations than the nuclear genomes do (Neiman & Taylor, 2009), and there are several factors that may be responsible for this. The mtDNA is more susceptible to oxidative damage due to its close

spatial proximity to ETC-mediated ROS generation sites in the mitochondria, the lack of protective histone proteins and its limited DNA repair mechanisms (Croteau & Bohr, 1997).

Moreover, somatic alterations in mtDNA may impair OXPHOS and enhance ROS production, thereby hastening the rate of DNA mutation (Tseng et al., 2006). As mitochondrial genome has only exons and no introns, any point mutation/ deletion could disrupt cellular respiration (Shamsi et al., 2008). Besides oxidative damage, other types of damage that can occur in mtDNA include alkylation damage, hydrolytic damage (formation of abasic sites and hydrolytic deamination of bases), formation of adducts, mismatched bases and DNA strand breaks (Alexeyev, Shokolenko, Wilson, & LeDoux, 2013).

It is generally believed that the important protective mechanisms and repair systems of the mtDNA are less robust than that of nuclear DNA, making it more vulnerable to damage. However, advances in the understanding of mtDNA repair processes demonstrate that mitochondria have most of the DNA repair pathways that the nucleus has (e.g. mitochondrial mismatch repair, long-patch base excision repair). Although more DNA repair enzymes with proven mitochondrial localisation have also been reported, detailed information on mitochondrial DNA repair is still lacking (Alexeyev et al., 2013).

3 | SPERM MITOCHONDRIA

3.1 | Morphological features

Fundamentally, the sperm mitochondrial structure and function are very similar to that of somatic cells. However, germ cell mitochondria are very dynamic and change morphologically and functionally throughout spermatogenesis (Zvetkova, Ilieva, Sainova, & Nikolov, 2018). The spermatogonia and early primary spermatocytes possess an 'orthodox' form of mitochondria. The late primary and secondary spermatocytes, and early spermatids harbour a more 'condensed' and metabolically more efficient form of mitochondria. The zygotene, late spermatids and mature spermatozoa have an intermediate-type mitochondria (Ramalho-Santos et al., 2009).

As the cytoplasm is being removed during differentiation of spermatids into spermatozoa (spermiogenesis), some mitochondria are removed in residual bodies leading to its reduced number. At

the end of spermatogenesis, typically 50–75 mitochondria remain tightly anchored around the nine outer dense fibres (ODFs) of the axoneme in the sperm midpiece (Figure 2). During spermiogenesis, they are organised end-to-end and wrap helically around the flagellum forming the thick mitochondrial sheath (Otani, Tanaka, Kasai, & Yoshioka, 1988). This capsule-like structure is made up of disulphide bonds between the cysteine- and proline-rich proteins. It functions to provide mechanical stability, and confer protection to sperm mitochondria (and mtDNA), as well as resistance to hypo-osmotic stress (Ramalho-Santos et al., 2009).

The anchoring of the mitochondrial sheath is maintained by a sub-mitochondrial reticulum (Olson & Winfrey, 1990). This appears to be dependent on the expression of the kinesin light chain 3 (KLC3) protein creating a bridge between the ODF1 and a mitochondrial outer membrane porin. Evidence from rodent experiments suggest that following epididymal maturation, sperm mitochondria become polarised and thereby functional (Aitken et al., 2007). A more loosely wrapped morphology of human sperm mitochondria is attained during capacitation, probably due to an expansion in mitochondrial volume (Vorup-Jensen et al., 1999).

3.2 | Sperm mitochondrial DNA

3.2.1 | Characteristics

There are approximately 70–80 mitochondria present in the midpiece of a single mammalian spermatozoon. The human spermatozoa harbour, on an average, one copy of mtDNA per mitochondrion (Ankel-Simons & Cummins, 1996). Moreover, sperm mitochondria possess an identical mtDNA sequence to that of somatic cells. However, DNA repair mechanisms in the sperm mtDNA seem to be either absent or less robust compared with that of somatic cells (Reynier et al., 1998), which predisposes the spermatozoa to rapid accumulation of mtDNA mutations. The mutation rate in sperm mtDNA is in fact about 10 to 100 times higher than that of nuclear DNA (Richter, Park, & Ames, 1988). The sperm mitochondria perhaps have no need for the repair or elimination of abnormal mtDNA, as it will not be transmitted during fertilisation. This also supports the need for maternal inheritance of the mtDNA (Hirata et al., 2002).

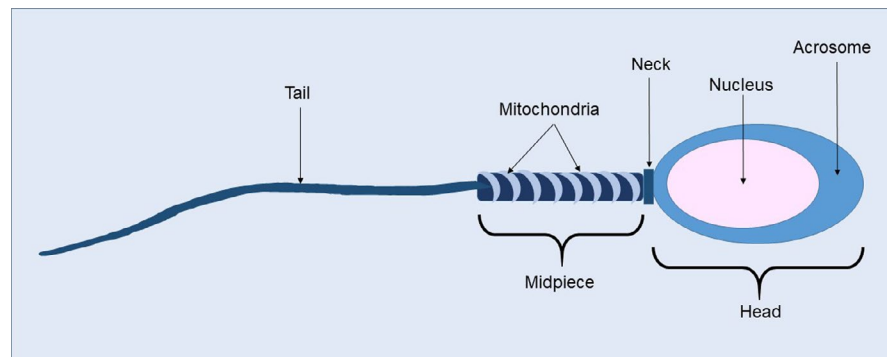


FIGURE 2 Diagram of human spermatozoa showing the spiral-shaped mitochondria in the midpiece

3.2.2 | Relationship between mitochondrial DNA integrity and sperm motility

The production of ATP is the most crucial aspect of mitochondrial function for supporting sperm motility (Piomboni, Focarelli, Stendardi, Ferramosca, & Zara, 2012). Ruiz-Pesini and colleagues were the first to show that the motility of human spermatozoa is entirely dependent on the functionality of the OXPHOS pathways (Ruiz-Pesini et al., 2000). They had earlier demonstrated that spermatozoa motility is directly correlated with activities of OXPHOS complexes I–IV (Ruiz-Pesini et al., 1998). Since sperm mtDNA partially encodes for OXPHOS-related proteins, any aberration in mtDNA may influence sperm motility.

Over the past two decades, studies have highlighted the relationship between mtDNA quality and sperm motility by investigating mutations, duplications and deletions in human sperm mtDNA (Amaral et al., 2013). These studies demonstrate that mtDNA point mutations, mtDNA single nucleotide polymorphisms (SNPs) and mtDNA haplogroups could greatly compromise semen quality (Kumar & Sangeetha, 2009). Infertile patients who had mtDNA diseases involving point mutations or multiple deletions of mtDNA were also identified to have asthenozoospermia or oligoasthenozoospermia (Wei & Kao, 2000). Novel point mutations of mtDNA have been reported in some spermatozoa with poor motility (Piomboni et al., 2012). The ratio of deleted mtDNA in spermatozoa with poor motility and subfertility was significantly higher than those in the motile, fertile spermatozoa (Kao, Chao, & Wei, 1998).

3.2.3 | Mitochondrial DNA transmission

During natural fertilisation, sperm mtDNA is not transmitted to the embryo in mammalian intraspecific crosses. Once the spermatozoon penetrates the oocyte, the paternal mitochondria and its DNA complement undergo degradation within the zygote and are eliminated during early embryogenesis before embryonic gene activation occurs (St John et al., 2010). Contrary to common belief that the sperm tail is discarded outside the oocyte during fertilisation, the entire male gamete does enter the oocyte (Ankel-Simons & Cummins, 1996; Ramalho-Santos, 2011). Human paternal mitochondria and mtDNA continue to persist until the 4- to 8-cell stage (St John et al., 2000).

Paternal mitochondria are then eliminated by a ubiquitin-mediated mechanism. This ensures a maternal-only mtDNA transmission which helps in maintaining homoplasmy (Wallace, 2007). The major protein of the inner mitochondrial membrane, prohibitin, is a potential target for ubiquitination (degradation signal) of sperm mitochondria (Sutovsky et al., 2000; Sutovsky, Van Leyen, McCauley, Day, & Sutovsky, 2004). Paternal mitochondria are modified by ubiquitin labelling during spermatogenesis and then degraded by proteasomes and/or lysosomes (Sato & Sato, 2012). Ubiquitin signals detected on sperm mitochondria within the male reproductive tract suggest that

sperm mitochondria are marked for selective degradation even prior to fertilisation (Sutovsky et al., 1999).

3.3 | Role of mitochondria in sperm function

Proper organisation of the ETC components is key in ensuring mitochondrial functionality, which is crucial for sperm function. Sperm mitochondrial function may be assessed by evaluating mitochondrial activity, MMP and calcium levels (Losano et al., 2018). Any insults that lead up to compromised sperm mitochondrial functionality could potentially impair sperm function (Amaral et al., 2013). Mice that are deficient of the testis-specific form of cytochrome c had a higher number of immotile spermatozoa and were less effective in fertilising oocytes (Narisawa et al., 2002).

Sperm mitochondrial oxygen consumption rate and mitochondrial respiratory efficiency were reported to correlate with sperm motility during capacitation *in vitro* (Stendardi et al., 2011). A peak in oxygen consumption was noted during *in vitro* capacitation and progesterone-induced acrosome reaction in boar spermatozoa (Ramio-Lluch et al., 2011). Several sperm mitochondrial proteins were found to undergo capacitation-dependent tyrosine phosphorylation (Shivaji, Kota, & Siva, 2009), indicating that mitochondrial functionality is required for sperm capacitation.

4 | MITOCHONDRIAL MEMBRANE POTENTIAL AS AN INDICATOR OF MITOCHONDRIAL FUNCTIONALITY AND SPERM QUALITY

Electron transfer along the respiratory chain is accompanied by the pumping of protons through the inner mitochondrial membrane, resulting in transmembrane differences in proton gradient. This proton-motive force drives the synthesis of ATP. Meanwhile, the protons flow passively back into the mitochondrial matrix via proton pores. Thus, ATP synthesis involves the coupling of electron transport and proton pumping to phosphorylation of ADP. The MMP is thereby generated by the proton pumps (i.e. complexes I, III and IV) during OXPHOS (reviewed in Ref. (Zorova et al., 2018)). In normal mitochondria, the transmembrane potential difference reflects the process of electron transport and OXPHOS that drives mitochondrial ATP production. Therefore, the MMP serves as a key indicator of mitochondrial activity and hence reflects sperm quality (Zhang et al., 2014).

The functional capabilities of spermatozoa can be correlated with mitochondrial functions, which are reflected by intact MMP (Agnihotri et al., 2016). Spermatozoa with low MMP are less capable of undergoing the acrosome reaction (Gallon, Marchetti, Jouy, & Marchetti, 2006; Zhang et al., 2019). Mitochondrial functionality by way of an intact MMP seems to be a pre-requisite in maintaining acrosin activity, acrosome reaction and chromatin integrity in human spermatozoa. Moreover, the dissipation of sperm MMP resulted in

an overproduction of ROS along with decreased ATP levels (Zhang et al., 2019).

Additionally, sperm MMP shows a direct and significant correlation with sperm count, normal morphology, motility and viability. Spermatozoa from young adults with oligozoospermia, asthenozoospermia and oligoasthenozoospermia were found to have significantly lower MMP than did normal spermatozoa, indicating that MMP plays a crucial role in sperm motility and fertility (Zhang et al., 2014). As the integrity of sperm MMP is an accurate reflection of sperm quality, its evaluation would be a useful complement to routine semen analysis (Espinoza, Schulz, Sanchez, & Villegas, 2009), particularly in the idiopathic infertile male (Barbagallo et al., 2020).

Sperm MMP is also considered a predictive factor of sperm fertilisation ability in both natural conception and in vitro fertilisation (IVF) (Kasai et al., 2002; Malic Voncina et al., 2016; Marchetti, Ballot, Jouy, Thomas, & Marchetti, 2012; Sousa et al., 2011). Regardless of semen quality, concurrent testing of both MMP and sperm DNA fragmentation was found to be better predictors of natural conception in infertile men (Malic Voncina et al., 2016).

5 | CONSEQUENCES OF SPERM MITOCHONDRIAL DYSFUNCTION

5.1 | Impaired sperm motility and DNA integrity

Defects in the mitochondrial ultrastructure in the midpiece of ejaculated spermatozoa have been implicated in cases of infertile men with persistent or severe unexplained asthenozoospermia (Mundy, Ryder, & Edmonds, 1995; Pelliccione et al., 2011). Thus, interruptions in energy production and sperm mitochondrial function may be an underlying cause of asthenozoospermia (Ferramosca, Provenzano, Coppola, & Zara, 2012).

Aerobic production of ATP by OXPHOS in the mitochondria is inevitably accompanied by the generation of ROS, mainly superoxide radicals and hydrogen peroxide (Munro & Treberg, 2017). Rotenone-induced inhibition of the respiratory chain complex I resulted in an increased production of H₂O₂ in the mitochondrial matrix via mechanisms independent of the MMP. This led to lipid peroxidation of the sperm midpiece and a subsequent loss of sperm motility. These effects were, however, reversed when the antioxidant α -tocopherol was administered concurrently with rotenone (Koppers, De Lullis, Finnie, McLaughlin, & Aitken, 2008). In oligoasthenozoospermic patients, incubation of spermatozoa with myo-inositol increased progressive motility and the number of spermatozoa retrieved after swim-up, which were associated with an increased proportion of spermatozoa with high MMP (Condorelli, La Vignera, Bellanca, Vicari, & Calogero, 2012).

Infertile males with poor sperm motility have a lower MMP compared with healthy fertile males. When treated with spermicidal agents, human spermatozoa showed a dramatic reduction in motility and subsequently a significant reduction in MMP. In addition, oxidative uncoupling of the mitochondrial ETC decreased both the MMP

and sperm motility (Agnihotri et al., 2016). These changes could have resulted from the abundant ROS released following the uncoupling of the ETC, which negatively impacts motility as well as the integrity of nuclear DNA (Treulen, Uribe, Boguen, & Villegas, 2015).

Low sperm MMP reflects sperm cells that are of low quality, which were associated with lower fertilisation rates in couples undergoing IVF (Marchetti et al., 2012). Despite MMP having a significant negative association with sperm motility, it, however, did not correlate with the fertilisation and pregnancy rates of infertile couples seeking ICSI treatment (Sharbatoghli, Valoerdi, Amanlou, Khosravi, & Jafar-abadi, 2012). Sperm motility is essential in IVF to gain access to the oocyte for fertilisation, but this is not the case in intracytoplasmic sperm injection (ICSI), in which the physiological barriers of spermatozoa entry into the oocyte are bypassed (Fisher & Henkel, 2020).

Molecular changes to mtDNA such as point mutations and deletions impact sperm motility and morphology, leading to reduced sperm functionality and male infertility (St John, Jokhi, & Barratt, 2005). For example, males with high levels of mtDNA point mutation A3243G were highly correlated with low sperm motility (Spiropoulos, Turnbull, & Chinnery, 2002). Furthermore, alterations in specific RNAs and transcripts of nuclear-encoded mitochondrial proteins have also been identified in sperm samples of asthenozoospermic patients (Jodar, Kalko, Castillo, Balleca, & Oliva, 2012). Aberrant expression of human mtDNA- and nuclear-encoded ETC proteins has been associated with compromised sperm quality (Amaral, Ramalho-Santos, & St John, 2007).

Sperm mitochondrial deletion is a marker of mtDNA integrity and damage. Males with poor semen parameters more commonly have sperm mitochondrial DNA deletions than normozoospermic men (Ieremiadou & Rodakis, 2009; Song & Lewis, 2008). While multiple mtDNA deletions are present in both testicular and ejaculated spermatozoa from the fertile and infertile men, however, a higher incidence of large-scale mtDNA deletions was observed in testicular spermatozoa of men with obstructive azoospermia. Large or multiple deletions suggest of a major disruption to the ETC, affecting ATP production and consequently sperm motility (O'Connell, McClure, & Lewis, 2002).

The common 4977-bp mtDNA deletion accumulates in various tissues during ageing and has been proposed as an mtDNA damage biomarker (Meissner et al., 2008). Males with asthenozoospermia, oligozoospermia and primary infertility were found to have a higher incidence of the 4977-bp mtDNA mutation. Moreover, intracellular levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG, a biomarker of oxidative DNA damage) were positively correlated with the level of mtDNA with 4977-bp deletions in spermatozoa (Kao, Chao, & Wei, 1995). Infertile asthenozoospermic and oligoasthenozoospermic males with large-scale 4977-bp deletions demonstrated heteroplasmic mtDNA (Ambulkar, Chuadhari, & Pal, 2016). An inverse correlation between the frequency of sperm 4977-bp mtDNA deletion and fertilisation rates was seen following IVF but not ICSI. This finding highlights the negative correlation between mtDNA integrity and sperm fertilisation efficiency (Ieremiadou & Rodakis, 2009).

In fact, both sperm mtDNA deletion rate and mtDNA copy number are associated with lower odds of fertilisation in an ART setting (Wu, Whitcomb, et al., 2019). Sperm mtDNA copy number (number of mtDNA copies per nuclear DNA copy) is also a sensitive biomarker of male fertility (Rajender, Rahul, & Mahdi, 2010). Human mtDNA copy number variations are associated with a decline of both sperm motility and fertility (Faja et al., 2019). An increase in mtDNA copy number along with a decrease in mtDNA integrity was observed in spermatozoa of infertile males with abnormal semen parameters (Song & Lewis, 2008). However, an earlier study found the copy number of mtDNA was lower in spermatozoa with poorer motility (Wei & Kao, 2000).

Comparative proteomic studies in asthenozoospermic males showed differential regulation of sperm proteins (Martinez-Heredia, de Mateo, Vidal-Taboada, Ballesca, & Oliva, 2008), including those associated with the fibrous sheath and with energy metabolism (Parte et al., 2012) as well as those concerning spermatogenesis and sperm maturation, sperm tail structure/motility and mitochondrial maintenance (Nowicka-Bauer et al., 2018). In addition, various proteins of intracellular trafficking, proteasomal proteins, heat shock proteins and tektins have been identified as differentially expressed in asthenozoospermic males (Amaral et al., 2014). Differential protein expression based on functional groups demonstrated that proteins of the 'energy and metabolism' groups were higher, while those of the 'movement and organisation' and 'protein turnover, folding and stress response' groups were lower in asthenozoospermic samples (Siva et al., 2010).

5.2 | Oxidative stress

Spontaneous generation of mitochondrial ROS was shown to cause a loss of MMP, lipid peroxidation, impaired sperm motility (Chai et al., 2017) and sperm DNA integrity. Thus, abnormal mitochondrial activity results in dysfunctional spermatozoa, which could then lead to male infertility (Koppers et al., 2008). There are several key pathways through which ROS may be generated by the sperm mitochondria, including disruption of the mitochondrial electron transport, formation of adducts with mitochondrial proteins, reduced mitochondrial expression of prohibitin, opening of the mitochondrial permeability transition pore (PTP) and induction of apoptosis in spermatozoa (reviewed in Aitken, 2018).

Increased levels of polyunsaturated fatty acids (PUFAs) trigger sperm mitochondrial generation of ROS. The dual hydrophilic and hydrophobic properties of PUFA (Aitken, Wingate, De Luliis, Koppers, & McLaughlin, 2006) facilitate its penetration of the inner mitochondrial membrane, disrupting the ETC electron flow, resulting in generation of superoxide anions and subsequently oxidative stress (Aitken, 2018). Consequent to this, peroxidative damage in the sperm tail impairs motility, eventually resulting in increased oxidative DNA damage (Koppers et al., 2008).

Once oxidative stress is initiated in the spermatozoa, ROS-induced generation of ROS occurs. Aldehydes generated as

by-products of lipid peroxidation (e.g. 4-hydroxynonenal) form adducts with mitochondrial proteins within the ETC, stimulating further production of mitochondrial ROS (Aitken et al., 2012). It also dysregulates sperm bioenergetic pathways, along with the structural and signalling machineries of the sperm tail (Nowicka-Bauer & Nixon, 2020).

The ensuing oxidative stress drives the spermatozoa along the intrinsic apoptotic cascade, from loss of MMP leading to oxidative DNA adduct formation, DNA fragmentation and ultimately cell death (Aitken et al., 2012). Increased generation of mitochondrial ROS induces the oxidatively stressed spermatozoa to undergo apoptosis via a truncated apoptotic pathway. Unlike that in somatic cells, chemical triggers of the apoptosis process are absent in spermatozoa. The spermatozoa will instead go down the apoptotic pathway by default unless prevented by pro-survival factors (Aitken, 2018).

Prohibitin is an important inner mitochondrial membrane protein that helps maintain the structural integrity of the ETC complexes. Prohibitin appears to regulate sperm motility by modifying the MMP and increasing ROS levels in infertile males with asthenozoospermia and/or oligoasthenozoospermia (Wang et al., 2012). A positive correlation between mitochondrial ROS and lipid peroxidation, as well as the negative correlation of mitochondrial ROS with prohibitin expression, high MMP and sperm motility, has also been reported in these groups of patients (Chai et al., 2017).

Increased intracellular calcium concentration leads to opening of the calcium-dependent pores in the sperm inner mitochondrial membrane, resulting in mitochondrial permeability transition that causes decreased MMP, increased ROS production and sperm DNA fragmentation (Treulen et al., 2015). Apoptosis in spermatozoa resulting from the generation of mitochondrial ROS is discussed in the following section 5.3. Additionally, exposure to both extremely low frequency and radiofrequency electromagnetic fields can also promote mitochondrial ROS production in spermatozoa (Santini et al., 2018), leading to formation of DNA base adducts, and subsequently DNA fragmentation (De Luliis, Newey, King, & Aitken, 2009).

High levels of reactive nitrogen species, such as peroxynitrate (a nitrogen oxide-derived oxidant), result in the generation of nitrosative stress. Nitric oxide radicals (present either by diffusion or via generation within the mitochondria) react with superoxide anions derived from the inner mitochondrial membrane to yield peroxynitrite. Peroxynitrite-induced formation of 4-hydroxynonenal protein adducts was found to increase as sperm motility decreased. Mitochondrial dysfunction leads to reduced motility in intact human spermatozoa, thereby contributing to male infertility (Cassina et al., 2015).

Infertile males with varicocele were reported to have underexpression of several ETC subunits, namely NDFSU1, UQCRC2 and COX5B. Moreover, *in silico* data revealed the underexpression of the principal mitochondrial proteins of structural organisation (e.g. LETM1, EFHC) and enzymes of the carbohydrate and lipid metabolic pathways. These findings suggest that there is metabolic dysregulation of sperm mitochondria in infertile males with varicocele (Samanta et al., 2018). Lower mitochondrial activity in varicocele

appears to be associated with sperm DNA fragmentation and acrosome integrity (Blumer et al., 2008, 2012).

5.3 | Apoptosis

Apoptosis is a programmed cell death mechanism that may be initiated through either the intrinsic (mitochondrial) pathway or extrinsic (death receptor) pathway. Both these pathways eventually activate the caspase cascade leading to protein degradation and cell death. Apoptosis is regulated by the pro-apoptotic BAX-BAK and anti-apoptotic BCL-2/BCL-XL respectively (Amaral et al., 2013). Not only are the mitochondria the site of interaction between anti-apoptotic and pro-apoptotic proteins, but it also originates the signals that initiate the activation of caspases via various mechanisms (Wang & Youle, 2009).

The intrinsic apoptotic pathway may be triggered by oxidative stress and/or high calcium levels, which induce the opening of the PTP (Amaral et al., 2013). Mitochondrial ATP synthase dimers are responsible for the formation of the PTP (Giorgio et al., 2013). Extrusion of cytochrome c through the PTP induces the activation of the caspase cascade and consequently leads to apoptosis (Amaral et al., 2013). As an example, prolonged exposure of human spermatozoa to phenylalanine resulted in mitochondrial superoxide generation and activation of intracellular caspase activity, thereby inducing apoptosis (Houston, Curry, & Aitken, 2015).

Activity of both caspases 3 and 9 was found to be localised in the ejaculated human sperm midpiece (Paasch, Grunewald, Agarwal, & Glandera, 2004; Weng et al., 2002). When apoptosis was induced in human spermatozoa, the activity of both caspases 9 and 3 increased, while MMP and sperm motility were decreased (Paasch, Grunewald, Dathe, & Glander, 2004). The activated caspases are associated with poor sperm quality, increased DNA fragmentation and lower fertilisation potential (Grunewald, Said, Paasch, Glander, & Agarwal, 2008; Weng et al., 2002). Along with the activation of caspase 3 and disrupted MMP, spermatozoa of infertile males were observed to have externalisation of the phospholipid phosphatidylserine and DNA fragmentation (Sakkas, Ramalingam, Garrido, & Barratt, 2015). Human sperm MMP is positively associated with semen volume, sperm concentration, count and progressive motility, but negatively correlated with apoptotic (Annexin V positive) spermatozoa (Zhang et al., 2016).

The phosphatidylinositol 3-kinase (PI3K)/AKT intracellular signalling pathway regulates the various cellular processes such as cell cycle progression, cellular growth, proliferation, survival and migration. Activation of the AKT (the core protein in the PI3K pathway) promotes cell survival, particularly under stress conditions (Szymonowicz, Oeck, Malewicz, & Jendrossek, 2018). Phosphorylation of AKT1 by the activation of the PI3K enzyme helps silence the downstream effectors of the apoptotic pathway such as Bcl-2-associated death promoter, thereby playing a vital role in maintaining spermatozoa in its functional state (Aitken, 2018).

However, when PI3K activity is inhibited, the AKT1 (RAC-alpha serine/threonine-protein kinase, an apoptosis inhibitor) rapidly dephosphorylates and spermatozoa enter the intrinsic apoptotic pathway. This results in caspase activation in the cytosol, excessive generation of mitochondrial ROS leading to significant loss of motility, as well as oxidative DNA damage (Koppers, Mitchell, Wang, Lin, & Aitken, 2011). However, since there is a clear structural separation between the nucleus in the sperm head and the mitochondria and cytoplasm at the sperm midpiece, the activated endonucleases are unable to gain physical access to cleave the nuclear DNA. Therefore, DNA fragmentation in human spermatozoa does not occur as a direct consequence of apoptosis, despite the presence of oxidative DNA adducts (Koppers et al., 2011).

6 | CONCLUDING REMARKS

Sperm mitochondria generate ATP and ROS required for physiological reproductive mechanisms, including motility, hyperactivation, capacitation and acrosome reaction. The functionality of the mitochondrial respiratory chain in spermatozoa is correlated with sperm quality and needs to be temporally maintained in mature spermatozoa up to fertilisation. Uncoupling of ETC decreases ATP production and increases ROS production leading to lipid peroxidation and low MMP. These changes result in a loss of sperm motility, poor sperm DNA integrity and lower fertilisation rates. The sperm mtDNA is vulnerable to oxidative damage owing to its proximity to the source of mitochondrial ROS production and lack of histones. Moreover, its limited DNA repair mechanisms predispose the spermatozoa to develop point mutations or multiple deletions of mtDNA that could compromise semen quality and impact fertility. Oxidative stress and leakage of cytochrome c drive the spermatozoa along the intrinsic apoptotic cascade, leading to the formation of DNA adducts and DNA fragmentation, resulting in apoptosis. Assessment of mitochondrial functionality may provide useful additional information in the management of male infertility.

AUTHOR'S PERSPECTIVE

Key points


1. Optimal mitochondrial functionality is fundamental for sperm motility, hyperactivation, capacitation, acrosome reaction and fertilisation.
2. Mitochondrial membrane potential is correlated with sperm quality, particularly sperm motility and DNA integrity.
3. Sperm mtDNA copy number and point mutation, duplication or deletion are associated with poor semen function leading to an increased risk of male infertility.
4. Sperm mitochondrial dysfunction is associated with the pathogenesis of seminal oxidative stress, a primary underlying cause of male infertility.

- Excessive generation of mitochondrial ROS leads to caspase activation and induces apoptosis, resulting in loss of motility and oxidative DNA damage.

Potential areas of research

- What is the basis for the transmission of the paternal mitochondrial genome into the zygote and its subsequent elimination? Are there other degradation mechanisms of the paternal genome involved besides ubiquitination?
- What is capacity of mature spermatozoa to mount a DNA repair response to oxidative stress and what are the mechanisms involved?
- What is the potential of mtDNA integrity to serve as an independent biomarker of sperm quality?
- What is the association between mitochondrial dysfunction and the impact of ageing on male fertility?
- Could the biological characteristics of sperm mitochondria serve as clinical biomarkers and diagnostic tools for sperm function and fertilising capacity?

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