

Mitochondria functionality and sperm quality

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Abstract

Although mitochondria are best known for being the eukaryotic cell powerhouses, these organelles participate in various cellular functions besides ATP production, such as calcium homeostasis, generation of reactive oxygen species (ROS), the intrinsic apoptotic pathway and steroid hormone biosynthesis. The aim of this review was to discuss the putative roles of mitochondria in mammalian sperm function and how they may relate to sperm quality and fertilisation ability, particularly in humans. Although paternal mitochondria are degraded inside the zygote, sperm mitochondrial functionality seems to be critical for fertilisation. Indeed, changes in mitochondrial integrity/functionality, namely defects in mitochondrial ultrastructure or in the mitochondrial genome, transcriptome or proteome, as well as low mitochondrial membrane potential or altered oxygen consumption, have been correlated with loss of sperm function (particularly with decreased motility). Results from genetically engineered mouse models also confirmed this trend. On the other hand, increasing evidence suggests that mitochondria derived ATP is not crucial for sperm motility and that glycolysis may be the main ATP supplier for this particular aspect of sperm function. However, there are contradictory data in the literature regarding sperm bioenergetics. The relevance of sperm mitochondria may thus be associated with their role in other physiological features, particularly with the production of ROS, which in controlled levels are needed for proper sperm function. Sperm mitochondria may also serve as intracellular Ca²⁺ stores, although their role in signalling is still unclear.

Reproduction (2013) **146** R163–R174

The mitochondrion: a multidimensional organelle

Mitochondria are important and unique organelles, and ongoing research keeps highlighting novel ways in which they participate in cellular functions. One main characteristic that separates the mitochondrion from other organelles is the presence of its own circular genome, mitochondrial DNA (mtDNA) and specific ribosomes, thus allowing for local protein synthesis (St John *et al.* 2010). Although mtDNA only codes for 13 mitochondrial proteins (Fig. 1), their expression might be essential for mitochondrial function. To this extent, mtDNA defects have been associated with a range of human disorders (including neurodegenerative diseases and cancer), as well as with ageing (for recent reviews, see Greaves *et al.* (2012) and Schon *et al.* (2012)). The development of animal models harbouring mtDNA mutations corroborated this association and contributed to the elucidation of mitochondrial disease mechanisms (Dunn *et al.* 2012).

In addition, mitochondria feature four defined interconnected compartments: the outer mitochondrial

membrane (OMM) and inner mitochondrial membrane (IMM), the intermembrane space and the mitochondrial matrix (Fig. 1). The similarities between the IMM and the cellular membrane of prokaryotic organisms (including the presence of the lipid cardiolipin), together with the existence of mtDNA, stress the possibility that mitochondria were once symbionts inside the cell, which progressively lost autonomy as most of their genome migrated to the nucleus, resulting in a full integration and control of mitochondria in eukaryotes (Alberts *et al.* 2008).

The IMM is usually convoluted, presenting several invaginations (cristae) but, unlike what is often assumed, this general arrangement is very variable, and the number, structure and extension of IMM cristae may have functional consequences (Bereiter-Hahn & Jendrach 2010). Furthermore, mitochondrial morphology itself is also plastic, with components of specific mitochondrial fission and fusion machineries promoting reversible changes from ovoid mitochondria to extensive interconnected filamentous organelles (Campello & Scorrano 2010). Finally, the functional importance of

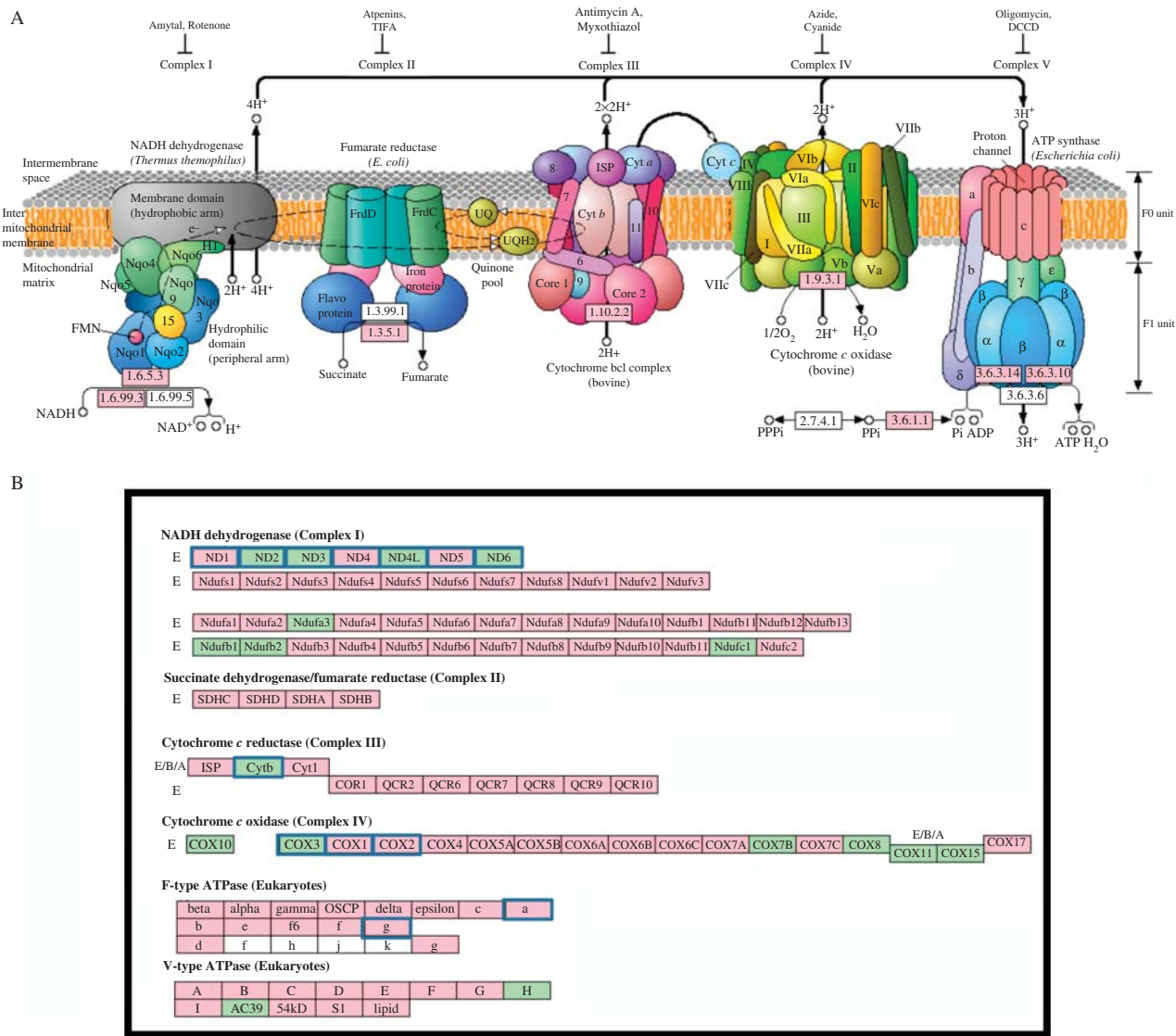


Figure 1 The mitochondrial electron transfer chain (ETC) and the production of ATP by oxidative phosphorylation. (A) The structure, composition and localisation of the ETC complexes is represented. Examples of inhibitors of each of the complexes are also indicated. (B) Proteins constituting each of the complexes. Adapted from the KEGG Pathway Database (<http://www.genome.jp/kegg/pathway.html>; Kanehisa *et al.* 2012). Details: pink rectangles, proteins described in human sperm proteomic studies (Amaral *et al.* 2013); green rectangles, proteins likely to be present but that have not been detected in human sperm proteomic projects; white rectangles, prokaryotic proteins; rectangles with blue frame, proteins encoded by the mitochondrial genome (all the others are nuclear encoded).

mitochondrial connections to other organelles (such as the endoplasmic reticulum) and the cytoskeleton is gaining attention, as it may help to integrate distinct cellular functions (Anesti & Scorrano 2006, Rowland & Voeltz 2012).

Mitochondria participate in many crucial processes in eukaryotic cells, the better known of which is the production of ATP via oxidative phosphorylation (OXPHOS), which is preceded by the generation of reduced electron carriers, both in the cytoplasm (via glycolysis) and in the mitochondrial matrix (where the Krebs cycle and the oxidation of most fatty acids take place, Fig. 2). The IMM includes several complexes that make up the electron transfer chain (ETC, Fig. 1A), which

transports electrons obtained from the oxidation of NADH and the FADH₂ moiety of succinate dehydrogenase, ultimately reducing the final acceptor oxygen to water. In this process, a quimio-osmotic proton gradient is generated across the IMM and is subsequently used by the ATP synthase to phosphorylate ADP to ATP. The proton gradient has two components, a minor chemical (pH) component and a major electric component, which is usually translated into the mitochondrial membrane potential (MMP). The electric nature of the MMP (with a negatively charged mitochondrial matrix) can also be harnessed to sequester calcium ions and thus participate in calcium homeostasis (Nichols & Ferguson 2002).

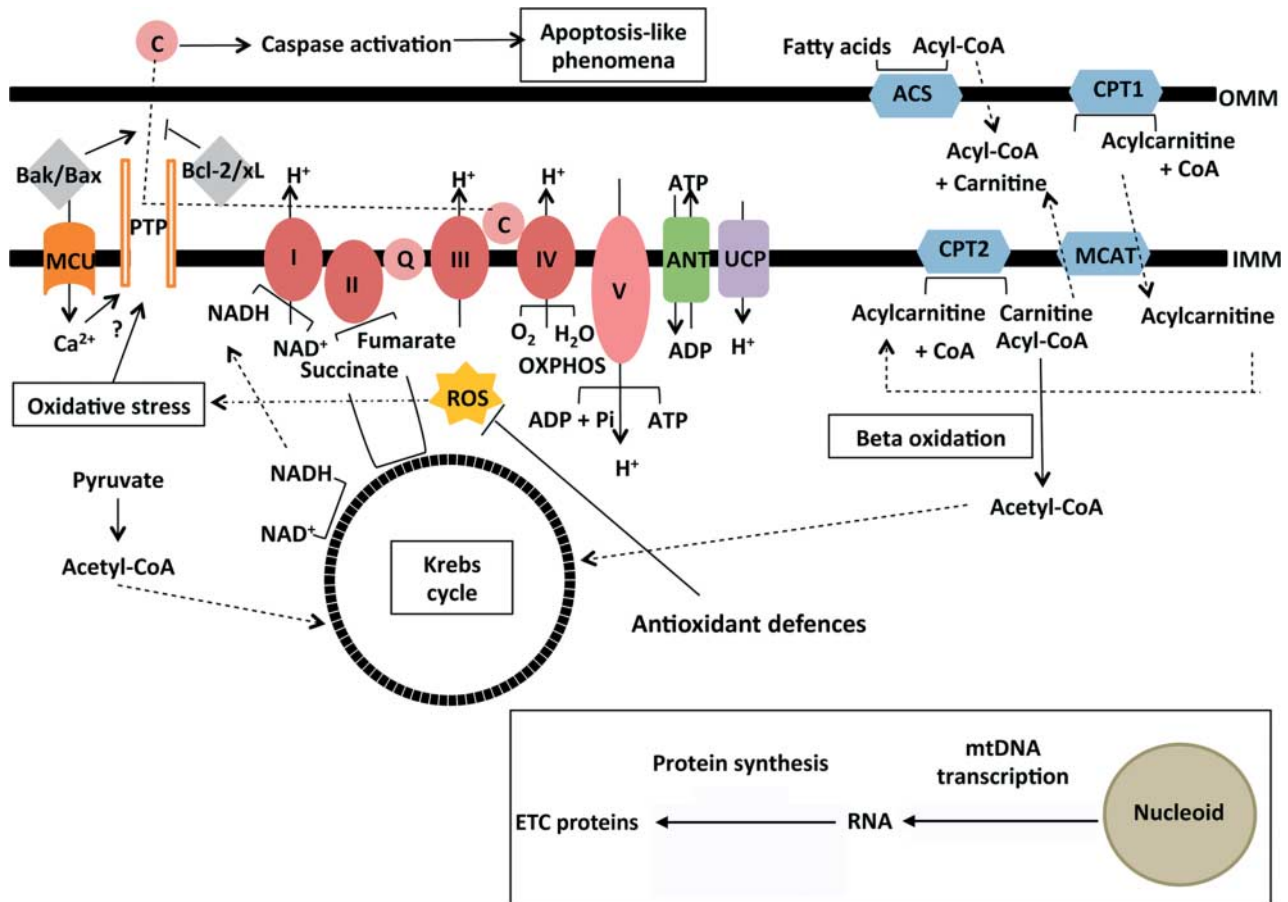


Figure 2 Overview of the pathways likely to be active in mammalian sperm mitochondria. Energy production by OXPHOS: the Krebs cycle and fatty acid β -oxidation contribute reducing equivalents to the electron transfer chain (ETC); ATP produced is exported from the matrix and ADP is imported. The proton (H^+) gradient may be dissipated by uncoupling proteins, under certain conditions. OXPHOS-derived ATP seems to be crucial for sperm function, although it does not seem to have a central role in sperm motility. Reactive oxygen species (ROS) production (which can be counteracted by antioxidant defences): controlled levels of ROS seem to be needed for sperm function; on the other hand, excessive levels may result in oxidative stress (and thus in DNA damage and lipid peroxidation). Intrinsic apoptotic pathway: oxidative stress and/or high Ca^{2+} levels can induce the opening of a permeability transition pore, the extrusion of cytochrome *c* and the activation of a caspase cascade, ultimately resulting in apoptosis-like phenomena. These may be stimulated/inhibited by apoptosis regulators (Bak–Bax and Bcl-2/xL respectively). Calcium uptake: although sperm mitochondria are known to uptake calcium, the role of sperm mitochondria in calcium signalling is unclear. mtDNA transcription and translation: the mtDNA is organized in protein complexes called nucleoids. Mammalian sperm mitochondria seem to have some protein synthesis activity. I, II, III, IV and V, ETC complexes; C and Q, electron carriers (cytochrome *c* and ubiquinone); ACS, acyl-CoA synthase; ANT, adenosine nucleotide translocator; CPT, carnitine acyltransferase; MCAT, mitochondrial carnitine/acylcarnitine carrier protein; MCU, calcium uniporter protein; PTP, permeability transition pore; UCP, uncoupling proteins.

Besides its involvement in ATP synthesis, the mitochondrial ETC promotes the production of reactive oxygen species (ROS), which can both function in signalling pathways and cause oxidative damage, if produced in an unchecked manner. Remarkably, the mobile ETC carrier cytochrome *c* moonlights as an active participant in the mitochondria-mediated intrinsic apoptotic pathway. In fact, one of the hallmark triggers of this process is cytochrome *c* release into the cytoplasm.

Importantly, for reproductive biology, mitochondria are also the starting point for steroid hormone biosynthesis (Ramalho-Santos & Amaral 2013). Indeed, the

conversion of cholesterol to pregnenolone (a common precursor for all steroid hormones) is catalysed by the cytochrome P450 side-chain cleavage enzyme (P450_{scc}) on the IMM (Stocco & McPhaul 2006). Moreover, mitochondrial ATP synthesis seems to be required for steroid biosynthesis in Leydig cells (Midzak *et al.* 2011). More recently, mitochondria and mitochondrial processes have been identified as participating in many other events, stressing its role in the integration of metabolism, cell signalling, cell proliferation, epigenetic regulation, cell cycle control, cell differentiation and cell death (Nunnari & Suomalainen 2012). Throughout this article, we will touch on several aspects of

mitochondrial functionality, specifically as they pertain to sperm function, and notably to human sperm, with brief mentions of research carried out in other species, as appropriate.

Sperm mitochondria

Germ cell mitochondria change throughout spermatogenesis: while spermatogonia and early spermatocytes harbour orthodox mitochondria, late spermatocytes, spermatids and sperm have more condensed (and metabolically more efficient) forms (see Ramalho-Santos *et al.* 2009). Additionally, concurrent to the loss of the majority of the cytoplasm occurring during spermiogenesis (the differentiation of spermatids into sperm), some mitochondria are lost in residual bodies. The 22–75 remaining mitochondria rearrange in tubular structures that are helically anchored around the anterior portion of the nine outer dense fibres (ODFs) and of the axoneme, constituting the midpiece (Otani *et al.* 1988, Ho & Wey 2007; Fig. 3). The anchorage of the mitochondrial sheath is sustained by a complex of filaments called sub-mitochondrial reticulum (Olson & Winfrey 1990) and seems to depend on the expression of

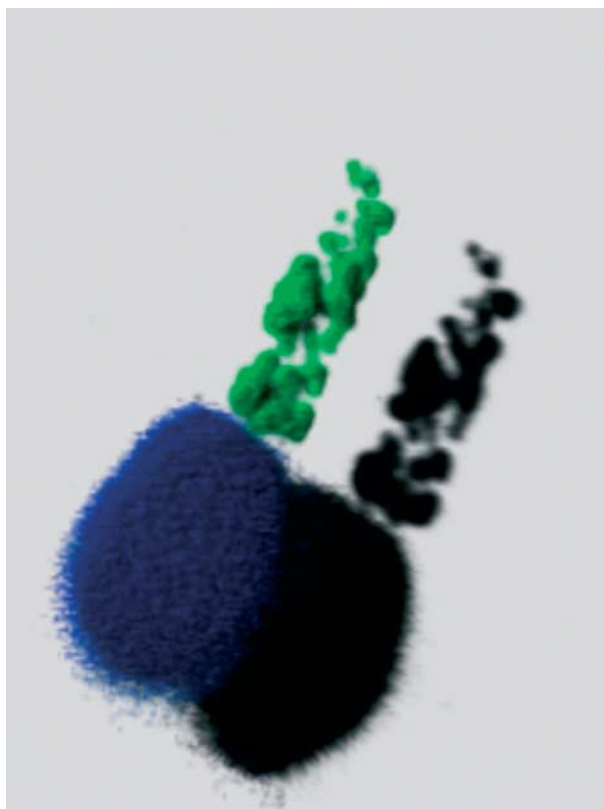


Figure 3 The human sperm midpiece. Three dimensional rendering of confocal microscopy images acquired with human sperm stained with an antibody against the mitochondrial protein TFAM, clearly showing the localisation and organisation of the sperm midpiece (green). DAPI was used as a DNA counterstain for the sperm nucleus (blue).

kinesin light chain 3 (KLC3), a protein that may bind both ODF1 and a mitochondrial outer membrane porin, creating a bridge between them. Indeed, transgenic male mice expressing a KLC3 mutant protein that cannot bind ODF1 have abnormal sperm midpiece formation, low sperm quality and reduced fertility (Zhang *et al.* 2012). On the other hand, the sperm OMMs are covered by a keratinous structure formed by disulfide bonds between cysteine- and proline-rich selenoproteins (Ursini *et al.* 1999). This structure, the so-called mitochondrial capsule, may confer protection to sperm mitochondria (and mtDNA) and certainly contributes to the impracticality of fully isolating these organelles. Logically, the number of mitochondrial proteins and of mtDNA molecules per cell is also reduced during spermiogenesis (Hecht *et al.* 1984, Larsson *et al.* 1997). However, given that most of the cytoplasm is lost during this differentiation process (thus greatly reducing cell volume), this may be paralleled by an increase in mtDNA copy number per volume unit (Diez-Sanchez *et al.* 2003).

Interestingly, sperm from a few non-mammalian animal species that live in habitats with very low oxygen levels lack mitochondria (Balsamo *et al.* 2007), suggesting that nature has a way of getting rid of needless mitochondria during spermiogenesis. On the contrary, mammalian sperm preserves a number of mitochondria in a specific subcellular compartment, indicating that the functionality of these organelles might be crucial. In addition, and at least in rodent species, it seems that sperm mitochondria become polarised, and thus functional, after epididymal maturation (without which sperm is unable to achieve *in vivo* fertilisation; Aitken *et al.* (2007)). Likewise, a remarkable change in human sperm mitochondria towards a more loosely wrapped morphology, possibly resulting from an increase in mitochondrial volume, was associated with capacitation (a second maturation process usually occurring in the female reproductive tract and without which *in vivo* fertilisation is not possible; Vorup-Jensen *et al.* (1999)). These observations suggest that active sperm mitochondria are required for fertilisation. From an evolutionary point of view, having more mitochondria may be advantageous, as sperm from primate species with multiple partners (and thus with stronger sperm competition) have a greater midpiece volume than sperm from monogamous species (Anderson & Dixon 2002).

Nevertheless, it is important to note that, although mitochondria are present in the male gamete, paternal mtDNA is generally not transmitted to the embryo in mammalian intraspecific crosses. However, despite what is depicted in many scientific textbooks, the reason for this maternal-only mtDNA transmission is not that the sperm tail is discarded outside the oocyte at fertilisation but rather that paternal mitochondria are degraded inside the zygote, following penetration of the entire male gamete into the oocyte (Ramalho-Santos 2011).

Having said this, the time frame during which sperm mitochondria functionality is physiologically relevant and needs to be maintained comprises the period between epididymal storage, ejaculation, travelling between the female reproductive tract and sperm–oocyte interactions. Any alteration in the mitochondrial genome, transcriptome, proteome or metabolome, or any cellular event resulting in compromised sperm mitochondrial functionality during this time may potentially affect sperm function, as will be discussed in detail (Table 1).

Mitochondrial functionality and sperm quality

First of all, defects in sperm mitochondrial ultrastructure seem to associate with decreased sperm motility in humans (Mundy *et al.* 1995, Pelliccione *et al.* 2011). At the molecular level, previous work has shown that deletions and other changes to mtDNA that influence cellular homeostasis can result in reduced sperm functionality and male infertility, both in human patients (for review see St John *et al.* (2005)) and in mice engineered to harbour a mutant mtDNA with a pathogenic 4696-bp deletion (Nakada *et al.* 2006). Likewise, microarray analysis suggested that sperm from asthenozoospermic samples have altered levels of specific mtRNAs, as well as of nuclear-encoded transcripts encoding mitochondrial proteins (Jodar *et al.* 2012). However, and at least for some mtRNAs, this putative difference could not be corroborated by quantitative real-time PCR. Moving beyond the mitochondrial genome/transcriptome, the expression of mitochondrial proteins, and notably ETC subunits, is associated with sperm quality (Amaral *et al.* 2007). In fact, comparative proteomic outcomes suggest that the expression of several sperm mitochondrial proteins may be altered in asthenozoospermic patients (Zhao *et al.* 2007, Martinez-Heredia *et al.* 2008, Chan *et al.* 2009, Siva *et al.* 2010, Parte *et al.* 2012). Furthermore, the activity of sperm mitochondrial enzymes, including ETC complexes, also correlates with sperm parameters, including concentration, vitality and motility (Ruiz-Pesini *et al.* 1998, 2000a), although it should be noted that the highest correlations found were for the activities of citrate synthase and ETC Complex II (succinate dehydrogenase), which are nuclear-encoded proteins that are part of the Krebs cycle. In addition, the normalisation of other activities to citrate synthase (often used as a marker for mitochondrial content) suggested that the main explanation for these correlations might be mitochondrial volume, not distinct enzymatic activities in samples of varying quality (Ruiz-Pesini *et al.* 1998). Additionally, mice lacking the testis-specific form of cytochrome *c* also have impaired sperm function (Narisawa *et al.* 2002). Furthermore, oxygen consumption in sperm mitochondria and mitochondrial respiratory efficiency also correlate with motility (Stendardi

et al. 2011, Ferramosca *et al.* 2012), and many different ETC inhibitors (Fig. 1) have been shown to negatively affect sperm motility (Ruiz-Pesini *et al.* 2000b, St John *et al.* 2005). Given that these results depend on an organized ETC (rather than on the activity of individual components), the data suggest that a functional organelle is important for sperm function. In accordance with this notion, the same should be valid for mitochondrial parameters that depend on intact mitochondria, namely the MMP.

Indeed, and although accurately monitoring MMP in sperm may be challenging (Amaral & Ramalho-Santos 2010), this parameter clearly correlates with functional sperm parameters, including motility (Troiano *et al.* 1998, Marchetti *et al.* 2002, 2004, Gallon *et al.* 2006, Paoli *et al.* 2011, Wang *et al.* 2012), and with fertilisation ability, monitored both in model systems (Sousa *et al.* 2011) and in patients undergoing assisted reproduction (Kasai *et al.* 2002, Marchetti *et al.* 2012). Interestingly, recent data suggest that the sperm motility of patients with abnormal sperm parameters can be enhanced by incubation with myoinositol, and this seems to be paralleled by an increase in the proportion of sperm with high MMP (Condorelli *et al.* 2012).

Finally, mitochondrial functionality may also be required for sperm capacitation. To this extent, a peak in oxygen consumption was observed during *in vitro* capacitation and progesterone-induced acrosome reaction in bovine and boar sperm (Cordoba *et al.* 2006, Ramio-Lluch *et al.* 2011). In addition, it is well established that several sperm mitochondrial proteins undergo capacitation-dependent tyrosine phosphorylation (for a review, see Shivaji *et al.* (2009)).

The preceding paragraphs seemingly stress that mitochondrial functionality is important for sperm activity, or, at the very least, that functional mitochondria help define a functional male gamete (Table 1). But what is exactly the role of mitochondria in sperm? Given that mitochondria are crucial for ATP production in eukaryotic cells and that ATP, in turn, is needed for sperm motility, the obvious answer would be to link these two events. However, the emerging portrait is much more complex, as will be discussed in the following section.

Sperm metabolism: not a linear story

In fact, the issue of sperm metabolism related to motility is the subject of an extensive debate (Ramalho-Santos *et al.* 2009), and some compelling evidence suggests that mitochondria-derived ATP is not paramount for motility, but rather that glycolysis may be the main ATP provider in this case, with mitochondrial activity at this level possibly related to other aspects. This hypothesis was first discussed in terms of compartmentalisation, namely that ATP produced in the midpiece would take too long to diffuse (or shuttle) along the flagellum, notably in species with longer sperm tails, such as rodents, although this

Table 1 Experimental evidence suggesting an association between mitochondrial functionality and sperm quality.

Mitochondrial feature	Main outcomes	References
<i>(A) Human sperm studies</i>		
Mitochondrial ultrastructure		
Midpiece and mitochondrial integrity	Sperm from asthenozoospermic patients have shorter midpieces and fewer mitochondrial gyres, disordered mitochondria with swollen intermembrane spaces, scattered disorganised cristae or a totally disaggregated inner structure (comparison with normozoospermic samples)	Mundy <i>et al.</i> (1995) and Pelliccione <i>et al.</i> (2011)
Mitochondrial genome (mtDNA)		
mtDNA rearrangements	Although conflicting results concerning specific point mutations/deletions were published, it seems consensual that the accumulation of multiple mtDNA rearrangements is associated with loss of sperm function	Reviewed in St John <i>et al.</i> (2005, 2007)
mtDNA content	Low-quality sperm have an abnormal mtDNA copy number	Diez-Sanchez <i>et al.</i> (2003), May-Panloup <i>et al.</i> (2003), Amaral <i>et al.</i> (2007) and Song & Lewis (2008)
Expression of proteins implicated in mtDNA maintenance	Low-quality sperm have lower levels of TFAM (mitochondrial transcription factor A) and POLG (DNA polymerase gamma)	Amaral <i>et al.</i> (2007)
Mitochondrial transcriptome (mtRNA)		
mtRNA levels	Sperm from asthenozoospermic patients have altered levels of specific mtRNAs (note: suggested by microarrays analysis but could not be corroborated by RT real-time PCR)	Jodar <i>et al.</i> (2012)
Mitochondrial proteome		
Protein levels	The expression of several mitochondrial proteins seems to be altered in sperm with low motility	Amaral <i>et al.</i> (2007), Zhao <i>et al.</i> (2007), Martinez-Heredia <i>et al.</i> (2008), Chan <i>et al.</i> (2009), Siva <i>et al.</i> (2010) and Parte <i>et al.</i> (2012)
Enzymatic activity	Correlation between the activity of ETC enzymes and sperm parameters (note: this may simply mirror the mitochondrial volume)	Ruiz-Pesini <i>et al.</i> (1998, 2000a)
Mitochondrial metabolism/bioenergetics		
ETC functioning and oxidation/phosphorylation coupling	Incubation of sperm with different ETC inhibitors results in decreased sperm motility	Ruiz-Pesini <i>et al.</i> (2000b) and St John <i>et al.</i> (2005)
MMP	Association between sperm MMP and sperm functional parameters (including motility) and fertilisation ability	Troiano <i>et al.</i> (1998), Marchetti <i>et al.</i> (2002, 2004, 2012), Wang <i>et al.</i> (2003, 2012), Gallon <i>et al.</i> (2006), Amaral & Ramalho-Santos (2010), Paoli <i>et al.</i> (2011) and Sousa <i>et al.</i> (2011)
Oxygen consumption and respiratory efficiency	Correlation between oxygen consumption/respiratory efficiency and sperm motility	Ferramosca <i>et al.</i> (2008, 2012) and Stendardi <i>et al.</i> (2011)
Others		
ROS production	Mitochondria are the main source of ROS in sperm	Koppers <i>et al.</i> (2008) and Kothari <i>et al.</i> (2010)
Apoptosis (intrinsic pathway)	Mitochondrial-derived ROS may induce an apoptosis-like phenomenon in sperm	Aitken <i>et al.</i> (2012c)
Ca ²⁺ signalling	Sperm mitochondria can uptake Ca ²⁺ and are possible intracellular Ca ²⁺ stores, but their role in signalling is unclear	Reviewed in Costello <i>et al.</i> (2009)
<i>(B) Genetically engineered mouse models</i>		
Mitochondrial mice models		
Testis-specific cytochrome c knock-out	Homozygous males were fertile, but presented testicular atrophy and their sperm were less motile, had lower levels of ATP and had a lower fertilisation ability compared with wild-types	Narisawa <i>et al.</i> (2002)
Mitochondrial DNA polymerase gamma (POLG) knock-in expressing a proofreading-deficient polymerase	Increased levels of mtDNA point mutations and deletions, reduced lifespan and premature onset of age-related phenotypes, including reduced fertility	Trifunovic <i>et al.</i> (2004)
Transmitochondrial (carrying mtDNA deletions)	Accumulation of pathogenic mtDNA-derived ETC defects;	Nakada <i>et al.</i> (2006)

notion is disputed (Ford 2006). There are, however, several lines of evidence that seem to favour glycolysis as the main ATP source for sperm movement. These include, for example, the need for glucose to maintain sperm function, a need that cannot be replaced

with OXPHOS substrates (Peterson & Freund 1970, Williams & Ford 2001, Amaral *et al.* 2011, Hereng *et al.* 2011). Furthermore, male mouse knock-out models for the glycolysis-associated enzymes enolase 4 (Nakamura *et al.* 2013), phosphoglycerate kinase 2

(Danshina *et al.* 2010), lactate dehydrogenase-C4 (LDHC; Odet *et al.* 2008) and glyceraldehyde 3-phosphate dehydrogenase-S (Miki *et al.* 2004) have impaired sperm function (notably in terms of motility) and suffer fertility loss, with the latter model maintaining normal mitochondrial activity. However, recent data have shown that, at least for LDHC, the severity of the results depends on the mouse strain, with some strains relying more on glycolysis than others (Odet *et al.* 2013). Using laser tweezers, it was also shown that human sperm motility was not dependent on MMP (Nascimento *et al.* 2008).

Therefore, it seems clear that there are contradictory data in the literature and that other metabolic pathways may be involved in sperm motility. Recent data suggest that the use of endogenous substrates, including the oxidation of fatty acids (Fig. 2), may be important for this process (Amaral *et al.* 2013), which should also be dependent on what physiological substrates and conditions the sperm encounters *in vivo* (Storey 2008).

Other aspects of mitochondrial physiology and sperm quality

ROS production

Sperm can be affected by ROS produced locally, or by ROS formed in leucocytes present in semen (Whittington & Ford 1999). Mitochondria are the main source of sperm-produced ROS, notably via the formation of superoxide in the ETC, although NADPH oxidase may also be an additional source (Koppers *et al.* 2008, Kothari *et al.* 2010). Importantly, controlled ROS levels are needed for proper sperm function (notably for motility, capacitation, the acrosome reaction, hyperactivation and fertilising ability), while ROS can also have a pathological effect on the male gamete, if in excess, or if there is an imbalance with available antioxidant defences, resulting in a decrease in viability, motility, MMP, and increases in DNA damage, morphology defects and lipid peroxidation, possibly resulting in apoptosis-like phenomena, as will be discussed below (Kothari *et al.* 2010, Mahfouz *et al.* 2010, Aitken *et al.* 2012b). The recent development of specific probes for mitochondria-produced ROS (mROS) shows that excessive production results in membrane peroxidation and loss of motility (Koppers *et al.* 2008, Aitken *et al.* 2012a). Additionally, a higher content of unsaturated fatty acid on sperm is also related to an increase in mROS again leading to motility loss and DNA damage (Koppers *et al.* 2010). Interestingly, mROS levels seem to vary in ejaculates, and when sperm are separated by Percoll gradients, the low-density fraction has a more prominent number of positive cells for mROS than the high-density fraction (Koppers *et al.* 2008, Aitken *et al.* 2013). These results have suggested that both enzymatic and non-enzymatic antioxidants could be used to control the

damage caused by excessive ROS levels in sperm, and there is some evidence that seems to substantiate this hypothesis, interestingly with antioxidants that specifically target mitochondria (Lamond *et al.* 2003, Aitken *et al.* 2012a).

Apoptosis

Although the capacity of mature sperm to carry out apoptosis has been questioned due to the paucity of cytoplasm, it is well known that human sperm can possess apoptotic markers and that this may influence sperm function and perhaps be involved in the removal of DNA-damaged sperm in the female reproductive tract (Ramalho-Santos *et al.* 2009, Aitken & Koppers 2011), or, alternatively, result from leftover apoptotic phenomena in the testis, possibly related to cases of male infertility (Almeida *et al.* 2013). It is worth mentioning that some studies note increases in sperm DNA damage as evidence for apoptosis, but, while DNA damage is certainly one of the main consequences of apoptosis, apoptosis may not be the only possible mechanism involved (Sousa *et al.* 2009, Aitken & De Luliis 2010, Sakkas & Alvarez 2010), and the notion of DNA damage directly linked to a canonical apoptosis cascade in sperm has been questioned (Koppers *et al.* 2011). Although the extrinsic apoptotic pathway has been suggested to be active in sperm (Sakkas *et al.* 1999), we will focus on the intrinsic (mitochondria-dependent) pathway, which involves, for example, both pro- and anti-apoptotic members of the Bcl family and especially on general apoptotic features, as there is clearly much more information at that level. In terms of the intrinsic pathway, anti-apoptotic Bcl-xL seems more prevalent in ejaculated abnormal/immature sperm, possibly as a spermatogenesis remnant (Cayli *et al.* 2004), while the presence of both pro- and anti-apoptotic forms of Bcl-x have been proposed to exist in mature human sperm, but no correlations with sperm parameters were shown (Sakkas *et al.* 2002).

More general apoptosis hallmarks include the externalisation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane and caspase activation. PS exposure can be monitored using fluorescent Annexin V in unpermeabilised (live) cells. In fact, Annexin V staining revealed more viable cells in normozoospermic patients (Varum *et al.* 2007) and seemed to correlate with sperm parameters in other studies (Shen *et al.* 2002, Weng *et al.* 2002). Importantly, the use of magnetic activated cell sorting (MACS) with Annexin V microbeads to select sperm reduced the percentage of altered cells (Lee *et al.* 2010, Rawe *et al.* 2010, Tavalaei *et al.* 2012). On the other hand, the presence of activated caspases (the final step in apoptosis) has also been linked to poor sperm quality and lower fertilisation potential, possibly by affecting sperm DNA (Weng *et al.* 2002, Grunewald *et al.* 2008, Kotwicka *et al.* 2008,

Almeida *et al.* 2011), both in the case of caspase 9 (activated by the mitochondrial pathway of apoptosis following cytochrome *c* release) and caspase 3 (activated by both apoptotic pathways). Interestingly, caspase activity seems to be focused in the sperm midpiece (Weng *et al.* 2002, Paasch *et al.* 2004a), and the use of apoptotic inducers increases the activity of both caspases, lowering MMP and sperm motility (Paasch *et al.* 2004b, Grunewald *et al.* 2005, Espinoza *et al.* 2009, Kim *et al.* 2012). Recent studies have implicated mitochondrial ROS generation in human sperm apoptosis, with resulting ROS-derived DNA damage rather than DNA cleavage, thus linking both phenomena (Aitken *et al.* 2012c). An interconnection between capacitation and apoptosis signalling pathways has also been proposed (Grunewald *et al.* 2009).

Ca²⁺ signalling

Calcium signalling and calcium store mobilisation have recently been shown to be important in Assisted Reproductive Technologies (ART) success, as responses are clearly different when patients are compared with sperm donors (Alasmari *et al.* 2013). However, what role sperm mitochondria have in this process is open to question, although sperm mitochondria are known to uptake calcium, and have been hinted as a possible intracellular calcium store in human sperm (Costello *et al.* 2009). In somatic cells, mitochondrial calcium uptake is undertaken by a mitochondrial calcium uniporter (MCU; Fig. 2) and is known to control intracellular calcium signals, cell metabolism and cell survival (for recent reviews, see Rizzuto *et al.* (2012) and Patron *et al.* (2013)). Proteomic data have confirmed that human sperm do possess MCU, as well as MCU regulator 1 (Amaral *et al.* 2013, Wang *et al.* 2013). However, mitochondrial uncoupling does not seem to significantly affect the calcium oscillations occurring in either progesterone- or nitric oxide-stimulated human sperm (Harper *et al.* 2004, Machado-Oliveira *et al.* 2008). Likewise, during bull sperm motility hyperactivation, mitochondrial respiration does not appear to be up-regulated by the release of calcium to the axoneme (Ho & Suarez 2003). Taken together, these data suggest that direct roles of mitochondrial calcium uptake in the control of intracellular calcium signals or in cell metabolism in mammalian sperm are unlikely. Mitochondrial calcium signalling may be involved in the sperm intrinsic apoptotic pathway, but further studies are needed to better clarify this aspect.

Conclusions and future perspectives

Although mitochondria functionality seems to be crucial for mammalian sperm, and while functional mitochondrial parameters clearly correlate with human

sperm functionality and fertilisation ability, its exact role in the male gamete is not completely clear (Fig. 2). At any rate, it seems that the specific and evolutionarily conserved mitochondrial concentration at the sperm midpiece of all mammalian species studied so far does not currently contribute towards centralising ATP production for sperm movement, as is often assumed in many Cell Biology textbooks (Alberts *et al.* 2008). Thus, the role of mitochondria in sperm function might be predominantly related to other physiological aspects. To this extent, on the one hand, the controlled production of mROS (balanced by effective antioxidant defences) seems to be required for sperm motility, capacitation and fertilising ability. On the other hand, the mitochondrial apoptotic pathway might prevent DNA-damaged sperm from participating in fertilisation and may also be linked to the removal of sperm from the female reproductive tract post-coitum (Aitken & Koppers 2011). Moreover, sperm mitochondria are putatively involved in Ca²⁺ homeostasis, as these organelles may function as intracellular Ca²⁺ stores (Costello *et al.* 2009), but more studies are required to better understand this topic. Additionally, similar to what seems to happen in other cells (Lu & Thompson 2012), a crosstalk between mitochondrial metabolism and sperm epigenetics may exist. This is especially relevant given the recent finding that the sperm chromatin may transfer acquired epigenetic states across generations (Puri *et al.* 2010).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

Funding

Part of the work in the authors' laboratory was funded by FEDER and COMPETE, via FCT (Fundação para a Ciência e Tecnologia), Portugal in grants PTDC/EBB-EBI/101114/2008, PTDC/EBB-EBI/120634/2010 and PTDC/QUI-BIQ/120652/2010. Center for Neuroscience and Cell Biology (CNC) funding is also supported by FCT (PEst-C/SAU/LA0001/2011). A Amaral is the recipient of a post-doctoral fellowship from FCT (SFRH/BPD/63120/2009).

Author contribution statement

B Lourenço and M Marques contributed equally to this work.

Acknowledgements

Given the extent of available literature, and the specific requirements of this review, readers have been referred to a few review articles. Apologies are due to all authors whose work was not directly cited. J Saints is acknowledged for proofreading the manuscript.

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Received 27 April 2013

First decision 5 June 2013

Revised manuscript received 6 July 2013

Accepted 30 July 2013