REVIEW ARTICLE

The role of mitochondria in energy production for human sperm motility

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Summary

Mitochondria of spermatozoa are different from the corresponding organelles of somatic cells, in both their morphology and biochemistry. The biochemical differences are essentially related to the existence of specific enzyme isoforms, which are characterized by peculiar kinetic and regulatory properties. As mitochondrial energy metabolism is a key factor supporting several sperm functions, these organelles host critical metabolic pathways during germ cell development and fertilization. Furthermore, spermatozoa can use different substrates, and therefore activate different metabolic pathways, depending on the available substrates and the physico-chemical conditions in which they operate. This versatility is critical to ensure fertilization success. However, the most valuable aspect of mitochondria function in all types of cells is the production of chemical energy in the form of ATP which can be used, in the case of spermatozoa, for sustaining sperm motility. The latter, on the other hand, represents one of the major determinants of male fertility. Accordingly, the presence of structural and functional alterations in mitochondria from asthenozoospermic subjects confirms the important role played by these organelles in energy maintenance of sperm motility. The present study gives an overview of the current knowledge on the energy-producing metabolic pathways operating inside human sperm mitochondria and critically analyse the differences with respect to somatic mitochondria. Such a comparison has also been carried out between the functional characteristics of human sperm mitochondria and those of other mammalian species. A deeper understanding of mitochondrial energy metabolism could open up new avenues of investigation in bioenergetics of human sperm mitochondria, both in physiological and pathological conditions.

Introduction

Mitochondria are sub-cellular organelles organized in four distinct sub-compartments. Starting from the outside, they present an outer mitochondrial membrane, an intermembrane space, an inner mitochondrial membrane and a matrix. The most specialized sub-compartments are the inner membrane and the matrix where many enzymes, generally organized as multi-subunit complexes, can be found. It has been reported that mitochondria contain about a thousand of distinct proteins involved in various metabolic pathways (Sickmann et al., 2003; Pagliarini et al., 2008). Moreover, mitochondria contain their own DNA (mtDNA), which encodes only a few mitochondrial proteins. The majority of mitochondrial proteins are therefore encoded by nuclear DNA. These proteins are synthesized on cytosolic ribosomes and post-translationally transported (or imported) into mitochondria where they are sorted to one of the four mitochondrial sub-compartments. The import of the cytosolically synthesized proteins into mitochondria requires a specialized machinery referred to as mitochondrial import machinery (Neupert & Herrmann, 2007; Chacinska et al., 2009; van der Laan et al., 2010). A strict and regulated cooperation between nuclear DNA and mtDNA is required to ensure a coordinated biogenesis of the mitochondrial multi-subunit complexes.

Mitochondria are generally known as the "power plant" of the cell because they play a fundamental role in

the production of ATP through the sophisticated mechanism of the oxidative phosphorylation or OXPHOS. Actually, even if this concept is largely true, we can consider it somehow reductive because many other metabolic pathways occur inside mitochondria, such as the citric acid cycle (or Krebs cycle), the oxidative decarboxylation of α-ketoacids, the β-oxidation of fatty acids, many reactions of the amino acid metabolism and of the pyrimidine synthesis. Furthermore, mitochondria are actively implicated in other processes, such as cell differentiation, ROS generation, apoptosis, calcium signalling, iron metabolism, etc. The complicated and fascinating process of mitochondrial OXPHOS, which represents the topic of this review, requires the coordinated operation of two main components, the respiratory chain and the ATP-synthase, both located in the inner mitochondrial membrane. The mitochondrial respiratory chain is involved in the transport of reducing equivalents from some electron donors to the molecule of O_2 with the final formation of H_2O. The respiratory chain uses the free energy released during this process for the generation of an electrochemical gradient of protons across the inner mitochondrial membrane. ATP-synthase uses this proton gradient for the synthesis of ATP. A strict coupling is therefore required between respiration and phosphorylation.

Mitochondria of sperm cells (sperm-type mitochondria) show peculiar characteristics. First of all, these organelles are exclusively confined in the sperm midpiece, tightly wrapped around the axoneme. The principal piece of the sperm tail is devoid of mitochondria and enriched in glycolytic enzymes (Eddy *et al.*, 2003; Krisfalusi *et al.*, 2006), many of which are distinct from the isoenzymes present in somatic cells (Welch *et al.*, 1992, 2000, 2006; Bunch *et al.*, 1998; Vemuganti *et al.*, 2007). Some of these enzymes, such as hexokinase, glyceraldehyde 3-phosphate dehydrogenase and aldolase, contain appropriate amino acid extensions at their N-terminus capable of anchoring the proteins to the fibrous sheath (Bunch *et al.*, 1998; Travis *et al.*, 1998, 1999; Vemuganti *et al.*, 2007). Secondly, sperm mitochondria are strictly associated to one another, thereby rendering difficult their isolation by conventional separation methods. The so-called mitochondrial capsule generated around the axoneme results from multiple disulphide bridges formed by a selenium-rich protein (Calvin *et al.*, 1981; Ursini *et al.*, 1999). This protein derives from the phospholipid hydroperoxide glutathione peroxidase that is enzymatically active in spermatids but becomes a structural protein in mature spermatozoa. Thirdly, sperm mitochondria possess specific isoforms of proteins and isoenzymes, such as cytochrome *c* (Goldberg *et al.*, 1977; Hess *et al.*, 1993), subunit VIb of the cytochrome *c* oxidase (Hüttemann *et al.*, 2003), lactate dehydrogenase (LDH-X or LDH-C4)

(Blanco & Zinkham, 1963; Goldberg, 1963) and E1-pyruvate decarboxylase (a component of the pyruvate dehydrogenase complex) (Gerez de Burgos *et al.*, 1994), which functionally differentiate sperm mitochondria from the somatic ones.

One of the main characteristics of sperm cells is their motility, which is required to ensure male fertility. Actually, there are two types of motility, the activated motility observed in the ejaculated spermatozoa and the hyperactivated motility, which is observed in spermatozoa at the site of fertilization. Both types of motility require an adequate supply of energy in the form of ATP that is used by the flagellar dynein-ATPase. A long-standing debate exists on which metabolic pathway, glycolysis or OXPHOS, is involved in energy production for sperm motility (Ford, 2006; Turner, 2006).

The scope of the present study was to propose an updated view of sperm mitochondrial bioenergetics that, especially from the comparison of mitochondria functionality in other tissues and in other species, will hopefully be able to suggest future directions of research in the area of human reproduction.

Mitochondrial oxidative phosphorylation

Excellent work has been published on the molecular mechanisms of OXPHOS and the reader is referred to it for an extensive and exhaustive overview of this topic (see the literature subsequently quoted in this paragraph). Here, we summarize a few general concepts that can be helpful for a better understanding of sperm mitochondrial bioenergetics. A particular emphasis is given to the flux of charged molecules across the inner mitochondrial membrane because this aspect is quite complicated, especially in sperm mitochondria, and is generally less known than the OXPHOS mechanisms.
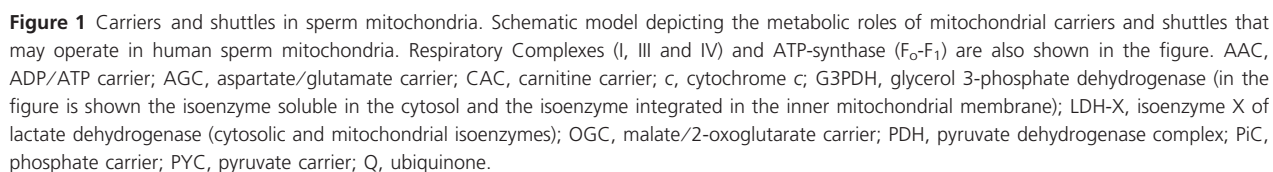
Metabolite flux across the inner mitochondrial membrane

To be functional, mitochondria require the intactness and impermeability of the inner membrane. The transit across this membrane is indeed impeded to almost all polar molecules, even to protons. At the same time, however, a continuous flux of charged molecules is necessary across the inner mitochondrial membrane to ensure an adequate supply of substrates for several metabolic events occurring inside or outside mitochondria. To fulfil this role, several protein carriers exist in the lipid bilayer of the inner mitochondrial membrane (Palmieri, 2004, 2008; Wohlrab, 2009).

The ADP/ATP carrier (or AAC) (Klingenberg, 2008) and the phosphate carrier (or PiC) (Krämer, 1996) are

strictly required for the synthesis of ATP inside mitochondria (Fig. 1). In fact, the continuous transport of ADP and Pi from the cytosol into mitochondria and the continuous efflux of matrix ATP towards the cytosol are required. Very interestingly, a novel AAC, besides that present in mitochondria, was recently identified in the fibrous sheath of the principal piece of human sperm (Kim *et al.*, 2007). This carrier presents unique properties, at first for its unusual localization outside mitochondria and then for its strict relationship with some glycolytic enzymes. These characteristics led the authors to propose an organization of the sperm fibrous sheath similar to that of the mitochondrial respiratory chain (Kim *et al.*, 2007).

Pyruvate, the final product of cytosolic glycolysis, is transported into mitochondria by the pyruvate carrier (or PYC) (Halestrap *et al.*, 1980; Hildyard & Halestrap, 2003) (Fig. 1). Once in the matrix, pyruvate is decarboxylated by pyruvate dehydrogenase (PDH), thereby generating acetyl-CoA, the principal fuel for the citric acid cycle (Fig. 1). The metabolism of pyruvate is, on the other hand, strictly correlated to that of L-lactate. In fact, L-lactate derives from the enzymatic reduction of pyruvate, especially in anaerobic conditions, and this reaction regenerates the cytosolic NAD^+ necessary for the progress of glycolysis. L-lactate produced in the cytosol of sperm cells can also be transported inside mitochondria for a further metabolization. It is generally thought that the mitochondrial uptake of L-lactate is a specificity of spermatozoa, but an active debate on this topic exists among researchers (Passarella *et al.*, 2008). The characteristics of the putative carrier responsible for the uptake of lactate inside sperm mitochondria are currently unknown. On the other hand, the existence of three distinct carriers for lactate has been proposed in somatic mitochondria (Passarella *et al.*, 2008). Furthermore, sperm mitochondria are not only able to internalize lactate but also capable of re-oxidizing it in the matrix to pyruvate (Fig. 1). This implies the presence of a novel isoenzymatic form of lactate dehydrogenase (LDH-X or LDH-C4) (Blanco & Zinkham, 1963; Goldberg, 1963), which is specific for sperm mitochondria. This isoenzyme is present in both the mitochondrial matrix and the cytosol of spermatozoa, with a net prevalence in this latter cellular sub-compartment (Alvarez & Storey, 1984; Burgos *et al.*, 1995). The joined operation of the lactate carrier and of the cytosolic and mitochondrial isoforms of LDH allows the transport of reducing equivalents from the cytosol into mitochondria (lactate/pyruvate shuttle, Fig. 1) (Gallina *et al.*, 1994). However, many aspects of lactate metabolism in spermatozoa remain to be elucidated.

The aspartate/glutamate carrier (AGC) and the malate/2-oxoglutarate carrier (OGC) cooperate together in the so-called malate-aspartate shuttle (Calvin & Tubbs, 1978; Burgos *et al.*, 1982) (Fig. 1). This shuttle, whose existence in sperm mitochondria of some mammalian

Figure 1 Carriers and shuttles in sperm mitochondria. Schematic model depicting the metabolic roles of mitochondrial carriers and shuttles that may operate in human sperm mitochondria. Respiratory Complexes (I, III and IV) and ATP-synthase (F_O-F_1) are also shown in the figure. AAC, ADP/ATP carrier; AGC, aspartate/glutamate carrier; CAC, carnitine carrier; c, cytochrome c; G3PDH, glycerol 3-phosphate dehydrogenase (in the figure is shown the isoenzyme soluble in the cytosol and the isoenzyme integrated in the inner mitochondrial membrane); LDH-X, isoenzyme X of lactate dehydrogenase (cytosolic and mitochondrial isoenzymes); OGC, malate/2-oxoglutarate carrier; PDH, pyruvate dehydrogenase complex; PiC, phosphate carrier; PYC, pyruvate carrier; Q, ubiquinone.

species has, however, been questioned, allows the transport of reducing equivalents from the cytosol into mitochondria with a mechanism more complicated than that previously seen in the case of the lactate-pyruvate shuttle (Fig. 1). Furthermore, mitochondria contain another shuttle, the glycerol 3-phosphate shuttle (Fig. 1), which, however, does not require a dedicated carrier protein in the inner membrane (Burgos *et al.*, 1982). Two isoenzymatic forms of glycerol 3-phosphate dehydrogenase (G3PDH), one soluble in the cytosol and the other bound to the inner mitochondrial membrane, are required for the operation of this last shuttle (Fig. 1).

Although the carnitine carrier (CAC) plays a fundamental role in mitochondria of somatic cells, its significance in sperm mitochondria has been underestimated or even questioned. In somatic mitochondria, CAC catalyses the internalization of long chain acyl-carnitine in exchange for free carnitine (Indiveri *et al.*, 1997) (Fig. 1). In the mitochondrial matrix, the molecules of acyl-carnitine regenerate acyl-CoA that is then addressed to the pathway of β-oxidation. In sperm mitochondria of some mammalian species, however, acyl-CoA, instead of acyl-carnitine, transit across the inner membrane thereby directly contributing to the intra-mitochondrial fatty acid catabolism (Carey *et al.*, 1981) (Fig. 1). The molecular mechanisms involved in this flux of acyl-CoA across the inner mitochondrial membrane are still unknown (Jeulin & Lewin, 1996). However, it has been found that human sperm mitochondria, as well as somatic mitochondria, are able to internalize acyl-carnitine (Ferramosca *et al.*, 2008).

Free carnitine, on the other hand, is critical inside sperm mitochondria because of its capability of buffering the acetyl units deriving from nutrient catabolism. In fact, an important stock of acetyl-carnitine was found in the mitochondrial matrix of sperm mitochondria from various mammalian species (Jeulin & Lewin, 1996) (Fig. 1). The metabolic significance of this reserve of acetyl-carnitine in sperm mitochondria and its interplay with acetyl-CoA are still under investigation.

Mitochondrial respiratory chain and ATP synthase

The mitochondrial respiratory chain is composed of four distinct multi-subunit complexes (Complex I, II, III and IV) and two electron shuttle molecules (ubiquinone and cytochrome *c*), which strictly cooperate in the transport of reducing equivalents from various electron donors to the molecule of oxygen (Fig. 2) (Lenaz & Genova, 2010). In addition, almost all respiratory complexes actively pump protons from the matrix into the intermembrane space, thus creating the mitochondrial proton-motive force. A large body of experimental evidence suggests that these complexes assemble as supra-molecular complexes, or super-complexes, in the inner mitochondrial membrane (Wittig *et al.*, 2006; Genova *et al.*, 2008).

The first and the largest component of the mitochondrial respiratory chain is Complex I or NADH dehydrogenase (Koopman *et al.*, 2010) (Fig. 2). Complex I catalyses the oxidation of the intramitochondrial NADH deriving, on the one hand, from the shuttles previously
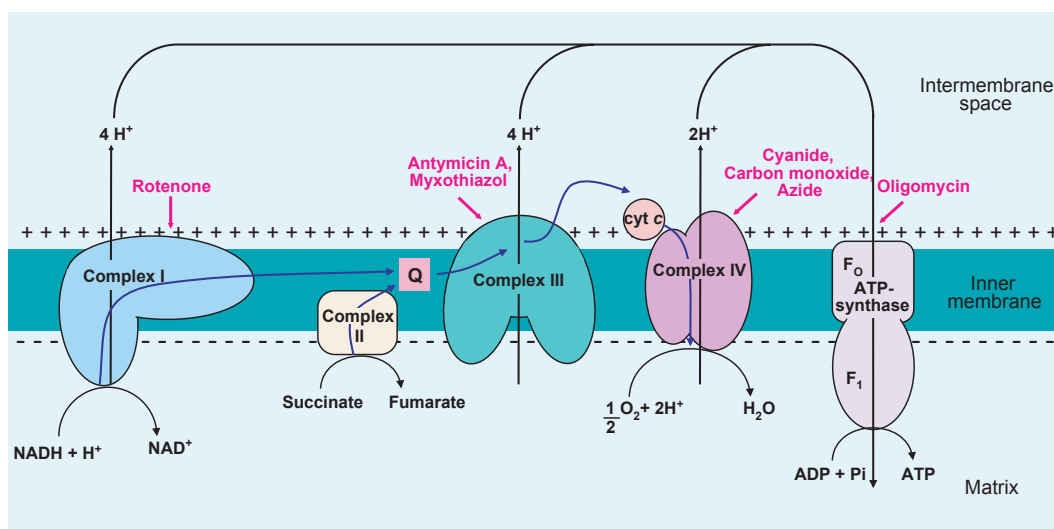

Figure 2 Oxidative phosphorylation system. Oxidative phosphorylation system is composed of five multimeric complexes. Electron transport from Complex I to Complex IV is coupled to extrusion of protons from the matrix into the intermembrane space at the level of Complexes I, III and IV. ATP-synthase synthesizes ATP using the free energy deriving from the physiological dissipation of the proton-motive force across the inner mitochondrial membrane. Some common mitochondrial respiratory chain inhibitors are shown. Cyt *c*, cytochrome *c*; Q, ubiquinone.

mentioned and, on the other hand, from several dehydrogenase reactions occurring inside mitochondria. Rotenone is a specific inhibitor of this mitochondrial respiratory complex (Fig. 2). Succinate, an intermediate of the citric acid cycle, represents the substrate of succinate dehydrogenase or Complex II (Rutter et al., 2010) (Fig. 2). Complex II does not transport protons into the intermembrane space and therefore does not contribute to the generation of the proton-motive force. Complex III or cytochrome bc_1 complex (Zara et al., 2009) is bigger than Complex II and operates as a homodimer in the inner mitochondrial membrane (Fig. 2). Complex III, which is inhibited by antimycin A and myxothiazol, channels the reducing equivalents to the small molecule of cytochrome c, a protein component of the respiratory chain not integrated in a multi-subunit complex. The last component of the mitochondrial respiratory chain is Complex IV, or cytochrome c oxidase (Fontanesi et al., 2008) (Fig. 2), which accepts reducing equivalents from cytochrome c and releases them to the molecule of O_2 forming H_2O. Complex IV is specifically inhibited by cyanide, carbon monoxide and azide (Fig. 2).

ATP-synthase is functionally connected to the mitochondrial respiratory chain (Devenish et al., 2008) (Fig. 2). This complex is made up of two main components, the F_o-ATPase inserted in the inner membrane, and the F_1-ATPase protruding in the matrix. The F_o-ATPase contains the channel through which protons return from the intermembrane space into the matrix, whereas F_1-ATPase catalyses the synthesis of ATP. ATP-synthase therefore synthesizes ATP using the free energy deriving from the physiological dissipation of the proton-motive force across the inner mitochondrial membrane (Boyer, 2001). Oligomycin, a strong and specific inhibitor of F_o-ATPase, blocks ATP synthesis and, consequently, mitochondrial respiration (Fig. 2). Finally, a supramolecular organization has been postulated also for ATP-synthase (Wittig & Schägger, 2009).

Mitochondrial energy metabolism in human sperm

Sperm cells, which undergo fundamental changes during spermiogenesis, maintain some mitochondria in the mid-piece whereas the rest of them and other unnecessary cellular components are completely removed. Mitochondria are in part saved because of their functional versatility that is required during the entire lifetime of human sperm, starting from spermatogenesis up to the final events of fertilization (Ramalho-Santos et al., 2009; Rajender et al., 2010). Furthermore, during spermatogenesis, mitochondria undergo significant modifications of their localization in the cell and of their structural and functional properties. The latter factors involve a profound change both in the number and size of mitochondrial cristae and an increase of respiratory activity (De Martino et al., 1979; Petit et al., 1995; Ruiz-Pesini et al., 2007).

Many studies on sperm mitochondrial bioenergetics carried out in humans, as well as in different mammals, have concentrated on the following question: what is the energy source for sperm motility, glycolysis or OXPHOS? Despite the various studies performed, still nowadays a conclusion cannot be drawn with certainty. What is the reason for this? First of all, the studies carried out in several species, including humans, have often provided different and/or conflicting results. Secondly, the experimental conditions varied significantly from one study to another and this complicated the interpretation of the results. Thirdly, the concept that glycolysis is an independent metabolic pathway inside sperm cells is incorrect. In fact, glycolysis is a preparative pathway that continuously supplies carbon units to mitochondria in which their complete oxidation occurs. Glucose is indeed metabolized into two molecules of pyruvate by glycolysis and during this process only two molecules of ATP/molecule of glucose are produced. Pyruvate, on the other hand, is further catabolized unless used in anabolic pathways. These pathways, however, do not, normally occur in spermatozoa because they are terminal cells. A general and reasonable concept that emerged from most studies is that sperm cells exhibit a great versatility in their metabolism using different mechanisms for energy production in dependence of the substrates available in the female genital tracts (Ruiz-Pesini et al., 2007; Storey, 2008).

Since the beginning of systematic studies on sperm bioenergetics (around the 1940s), many authors reached the conclusion of a primary and/or exclusive role of glycolysis in energy production for human sperm motility (for a review see Storey, 2008). In these experiments, it was difficult to even identify the mitochondrial cytochromes that, however, were successfully recognized some years later. The concept of a high glycolytic activity and a low mitochondrial OXPHOS in human spermatozoa, on the other hand, was also confirmed later by other researchers (Peterson & Freund, 1970, 1974; Storey, 1978; Ford & Harrison, 1981; Williams & Ford, 2001). In most of these experiments, mitochondrial functionality was assayed by measuring the oxygen uptake by intact human sperm cells polarographically. The addition of various respiratory substrates, such as pyruvate, malate, fumarate, citrate, 2-oxoglutarate and pyruvate/oxaloacetate, to intact sperm cells did not increase the mitochondrial oxygen consumption over the endogenous rate, thereby leading to the conclusion that OXPHOS in human spermatozoa is less efficient than glycolysis (Peterson & Freund, 1970).

A certain stimulation of respiration was only seen after the addition of succinate, a substrate of Complex II of the respiratory chain (Peterson & Freund, 1970; Ford & Harrison, 1981). It was also suggested that this inefficient OXPHOS was not caused by a reduced activity of the citric acid cycle enzymes (Peterson & Freund, 1974). Instead, the competitive effect of the intramitochondrial LDH-X for the electrons generated by the dehydrogenase reactions of the Krebs cycle was considered as one of the possible reasons for the scarce mitochondrial respiration found in human sperm. Moreover, a lower activity of Complex IV was detected in human sperm mitochondria in comparison to rat sperm mitochondria (Ford & Harrison, 1981).

The use of intact spermatozoa for the assay of mitochondrial functionality is, however, not devoid of serious drawbacks. In fact, the addition of various substrates to intact sperm cells does not ensure that these molecules are really delivered to mitochondria. In addition, almost nothing is known on the amount of endogenous substrates that can be oxidized by mitochondria and that are already present in the cytosol of intact human spermatozoa. Furthermore, samples of intact spermatozoa used in these experiments may be contaminated by other cellular types or may contain sperm aggregates. On the contrary, the studies carried out on somatic mitochondria employed pure organelles in rigorous and standardized experimental conditions.

Given the difficulty in isolating mitochondria from sperm mid-piece, a methodology originally developed for animal studies (Keyhani & Storey, 1973; Calvin & Tubbs, 1978; Storey, 1980; Carey et al., 1981; Piasecka et al., 2001) has recently been applied in human samples (Ferramosca et al., 2008). Briefly, human spermatozoa were incubated in hypotonic buffer to selectively disrupt the plasma membrane and were subsequently used for studies of mitochondrial respiration. With this treatment, mitochondria maintained their intactness and functionality, but at the same time, any physical obstacles to the diffusion of the respiratory substrates to the mitochondrial membranes were removed. The hypotonically treated human sperm mitochondria respired very efficiently in the presence of pyruvate/malate (Ferramosca et al., 2008), accordingly to previous results (Hutson et al., 1977; Storey & Kayne, 1977, 1978; Gerez de Burgos et al., 1994; Piasecka et al., 2001), as malate has a stimulatory role on pyruvate oxidation. In these samples, the ratio between the rate of oxygen uptake in presence of these substrates plus ADP (V_3) and the rate of oxygen uptake in presence of the substrates alone (V_4), allowed for the calculation of a respiratory control ratio (or RCR) of about 2.5, hence indicating a good coupling between respiration and phosphorylation. The absolute value of V_3, corresponding to

45 nmol $O_2 \times mL^{-1} \times min^{-1}/10^8$ cells (Ferramosca et al., 2008), was much higher than previously reported for intact human spermatozoa (Ford & Harrison, 1981). In hypotonically treated human samples mitochondria respired also in the presence of other oxidable substrates and retained a full sensitivity to various inhibitors. Interestingly, the finding that human sperm mitochondria respired also after the addition of lactate plus malate (Ferramosca et al., 2008) supports the view that mitochondria are able to internalize and oxidize lactate. Furthermore, demembranated human spermatozoa respired very efficiently with palmitoyl-carnitine, but not with palmitoyl-CoA. However, the addition of free carnitine to these samples, subsequently to palmitoyl-CoA, restored respiration. These results, although supporting the existence of an active carnitine palmitoyltransferase in human sperm, differ from those obtained in sperm mitochondria from other mammals in which a direct transport of acyl-CoA was found (Carey et al., 1981).

Other authors underlined the importance of mitochondrial OXPHOS for sperm motility and quality assaying mitochondrial functionality with other approaches, such as spectrophotometric methods, or using potential-sensitive dyes (Evenson et al., 1982; Auger et al., 1989, 1993; Kramer et al., 1993; Ruiz-Pesini et al., 1998, 2000; Marchetti et al., 2002; Gallon et al., 2006; Espinoza et al., 2009; Sousa et al., 2011). It has been proposed that the membrane potential ($\Delta\psi$) across the inner mitochondrial membrane of human spermatozoa is an indicator of sperm quality, ensuring, when sufficiently high, a proper sperm motility and a good fertilizing ability (Troiano et al., 1998; Donnelly et al., 2000; Marchetti et al., 2002; Espinoza et al., 2009).

The importance of mitochondrial functionality was also extended to hyperactivated motility and to the strictly associated phenomenon of sperm capacitation (Hicks et al., 1972; Williams & Ford, 2001). The mitochondrial respiratory efficiency was therefore investigated in swim-up selected human spermatozoa, incubated under capacitating conditions, in comparison to control samples. The oxygen uptake was eventually determined in these samples after hypotonic swelling. An impressive increase in the value of V_3 (i.e. in the presence of respiratory substrates plus ADP) was found in the swim-up selected and capacitated samples in comparison to the control ones (Stendardi et al., 2011). This suggests that capacitation of human spermatozoa is accompanied by a strong increase in mitochondrial functionality. Furthermore, in these samples, the polarographic assay of oxygen consumption was carried out with just 200 000 spermatozoa/mL, a level of sensitivity never reached before.

The measurement of sperm swimming speed and swimming force along with $\Delta\psi$ using the combination of

laser tweezers, fluorescent imaging and real-time auto-mated tracking and trapping confirmed, however, the primary role of glycolysis in energy production for human sperm motility (Nascimento *et al.*, 2008). Indeed, no relationship was found between the sperm motility and mitochondrial membrane potential. In addition, the effects of glucose and of various OXPHOS and glycolytic inhibitors on the sperm motility and mitochondrial $\Delta\psi$ suggested that OXPHOS alone is unable to sustain high motility in the presence of glucose.

In our opinion, a first conclusion that can be drawn from all these findings is essentially of methodological nature. Although in the case of somatic mitochondria, and in particular of liver mitochondria, there exist well-standardized conditions under which the experiments are carried out, this is true only in part in the case of sperm mitochondria. Sperm mitochondria are clearly different, both structurally and functionally, from somatic mitochondria and this implies dedicated experimental approaches. It is therefore possible that a methodological problem generates a conceptual problem, that is, the difficulty to rationalize the results in the functional context of spermatozoa.

Human asthenozoospermia: structural alterations of sperm flagella

Asthenozoospermia is a fertility-impairing pathology linked to a more or less pronounced reduction of sperm motility. Structural alterations of sperm flagella are responsible for reduced motility in asthenozoospermic infertile men. The complexity of these alterations can be determined only at an ultrastructural level by electron microscopic analysis of semen samples. Using this approach, defects in the components of the periaxonemal structures, mitochondrial helix and fibrous sheath, can be recognized together with alterations in the number and organization of the axonemal microtubules (Courtade *et al.*, 1998). Two kinds of tail abnormalities have been detected in men with severe asthenozoospermia: phenotypic and genotypic alterations (Baccetti *et al.*, 2001) (Table 1).

Phenotypic alterations are flagellar anomalies found in a variable percentage of spermatozoa in different samples. These alterations result from several pathologies, such as varicocele, microbial infections or hormonal unbalance, and are amenable to pharmacological or surgical remedies in order to retain fertility.

In human spermatozoa, the mitochondrial sheath is organized in a helix of about 13 gyres, with two mitochondria *per gyrus*, surrounding the axoneme at the mid-piece level. Abnormalities of mitochondrial organization include a shorter mid-piece with fewer mitochondrial gyres (Mundy *et al.*, 1995), total absence of mitochondria from the mid-piece, lack of the mid-piece segment, bad assembly or clustering of mitochondria with normal ultrastructure (Wilton *et al.*, 1992; Gopalkrishnan *et al.*, 1995). Ultrastructural mitochondrial changes include increased matrix, thickening of membranes, and parallelization of cristae and lipid inclusions which may also appear in spermatids. This last finding therefore suggests that all these modifications represent a primary defect rather than a secondary one because of degeneration of spermatozoa (Folgero *et al.*, 1993). When a multidisciplinary approach including immunocytochemistry and molecular techniques is applied, a more precise characterization of

Table 1 Structural anomalies of sperm mitochondria

Authors	Defect	Clinical implications
Phenotypic alterations		
Wilton *et al.*, 1992	Few mitochondria, or altered mitochondria	Asthenozoospermia
Mundy *et al.*, 1995	Few gyres of mitochondria	Asthenozoospermia
Gopalkrishnan *et al.*, 1995	Abnormal mitochondria	Severe asthenozoospermia
Piasecka & Kawiak, 2003	Alterations in mitochondrial sheath which contains functional mitochondria	Asthenozoospermia
Genotypic alterations		
Dysplasia of the fibrous sheath		
Rawe *et al.*, 2001	Few and disorganized mitochondria	Severe asthenozoospermia or total immotility
Sutovsky *et al.*, 2001	Few and disorganized mitochondria	Severe asthenozoospermia or total immotility
Rawe *et al.*, 2002	Few and disorganized mitochondria	Severe asthenozoospermia or total immotility
Absence of the fibrous sheath		
Ross *et al.*, 1971	Elongation of mid-piece	Few motile spermatozoa
Baccetti *et al.*, 2004	Abnormal extension of the mitochondrial helix	Few motile spermatozoa

mitochondria abnormalities, including structural, molecular and functional aspects, should be expected. Indeed, Piasecka & Kawiak (2003) evaluated different parameters such as the changes in mitochondrial membrane potential and mass of mitochondria by flow cytometry using specific probes (JC-1 and Mito Tracker Green FM respectively), the oxidoreductive capability of sperm mitochondria using cytochemical reactions for NADH-dependent dehydrogenases and the morphology of the mid-piece by light, fluorescent and electron microscope. These authors pointed out that in some cases of asthenozoospermia, sperm mitochondria can be functionally active and present a high $\Delta\psi_m$ in a large number of cells. Therefore, the low sperm motility does not necessarily result from energetic disturbances of sperm mitochondria, but it may be associated with deformations of the mitochondrial sheath containing functional mitochondria.

Genotypic alterations of the tail are monomorphic, primary anomalies affecting the whole sperm population during the entire life. Differently from phenotypic alterations, they cannot be corrected with pharmacological or surgical treatments. These structural anomalies cause a complete absence of motility or a minute beat of the sperm flagellum.

The genotypic sperm defect DFS (dysplasia of the fibrous sheath) (Chemes et al., 1987, 1998), associated with respiratory pathology and familial inheritance, is characterized by different distortions in the fibrous sheath, microtubular doublets and mitochondrial sheath, all arranged in a short and thick tail. In DFS, the annulus does not migrate caudally and is retained just below the connecting piece; therefore, mitochondria do not assemble in a normal mid-piece (Rawe et al., 2001). DFS spermatozoa also show increased mitochondrial and surface ubiquitination (Sutovsky et al., 2001; Rawe et al., 2002), suggesting a mechanism of selective elimination of defective spermatozoa.

The absence of the fibrous sheath (Ross et al., 1971; Baccetti et al., 2004) is a monomorphic sperm defect characterized by a structurally abnormal tail with a very elongated mid-piece showing supernumerary mitochondrial gyres. The principal piece is devoid of the fibrous sheath, and extra-axial microtubules come out at any level of the flagellum.

In the modern era of assisted reproduction, it is possible, in some instances, to overcome the problems of sperm immotility, even in the cases due to genetic tail defects, by intracytoplasmic sperm injection (ICSI). An evaluation of the ultrastructural sperm quality was performed in males attempting assisted reproductive procedures (Baccetti et al., 2002) by comparing three parallel investigations on in vitro fertilizations (IVF), ICSI and partial zone dissection (PZD). In this study, the fertilization rate using the three different techniques was correlated with normal mitochondrial structure and helix assembly.

On the other hand, Rawe et al. (2007) reported that mid-piece defects, while causing serious motility alterations and lower fertilization potential, are anyhow capable of normal fertilization using ICSI. Indeed, the obtained embryos had a good implantation potential. However, although a genetic origin of these mid-piece anomalies has been suggested, a genetic counselling will not be possible until the genes involved and the mode of inheritance are fully understood. Furthermore, it has recently been reported that structural defects in mitochondrial membranes are strongly associated with unexplained human asthenozoospermia (Pelliccione et al., 2011).

Human asthenozoospermia: functional mitochondrial alterations

The role of mitochondria in the aetiology of asthenozoospermia is far from being clear mainly because the cause of several cases of this pathology is still unknown. However, by using different methodological approaches, several authors demonstrated the presence of defects in the mitochondrial respiratory activity in idiophatic as well as

Table 2 Defects of mitochondria functionality

Authors	Method	Results	Clinical implications
Evenson et al., 1982	Determination of mitochondrial membrane potential ($\Delta\psi_m$)	Reduced $\Delta\psi_m$	Asthenozoospermia
Ruiz-Pesini et al., 1998	Determination of enzimatic activity by spectrophotometric assay	Reduced activity of Complexes I, II and IV and of citrate synthase	Asthenozoospermia
Ruiz-Pesini et al., 2000	Determination of enzimatic activity by spectrophotometric assay	Reduced activity of Complex II of citrate synthase	Oligozoospermia
Marchetti et al., 2002	Determination of mitochondrial membrane potential ($\Delta\psi_m$)	Reduced $\Delta\psi_m$	Asthenozoospermia
Ferramosca et al., 2008	Determination of oxygen consumption by polarographic assay	Reduction of respiratory control ratio	Asthenozoospermia

in varicocele-related cases of asthenozoospermia (Table 2).

The first article relating mitochondrial membrane potential to sperm motility dates back to almost 30 years ago (Evenson *et al.*, 1982). These authors reported a good correlation between sperm motility and mitochondrial membrane potential ($\Delta\psi_m$) measured with the fluorochrome Rhodamine 123 when comparing ejaculates from fertile men with those from patients whose spermatozoa showed reduced sperm motility. With the same technique but applying the methodology to a larger number of semen characteristics and using a different fluorochrome (DIOC$_6$), Marchetti *et al.* (2002) confirmed that a high $\Delta\psi_m$ is positively correlated with standard semen parameters such as motility. Later, the same group expanded their observations and concluded that there is a good correlation between $\Delta\psi_m$ and overall fertilizing capability of spermatozoa in human ejaculated samples (Gallon *et al.*, 2006). Very recently, it has been demonstrated that a subpopulation of human spermatozoa showing a better fertilization potential is characterized by a high mitochondrial functionality, as assessed with mitochondrial-sensitive probes (Sousa *et al.*, 2011).

Ruiz-Pesini *et al.* (1998, 2000) determined the activities of some mitochondrial enzymes (citrate synthase and respiratory Complexes I, II, I+III, II+III and IV) spectrophotometrically and correlated them with distinct seminal parameters, in particular with sperm cell motility. The authors not only found a high correlation between sperm motility and mitochondrial enzyme activities, but also a strict dependence of this motility on mitochondrial volume (Ruiz-Pesini *et al.*, 1998). Furthermore, we recently demonstrated a significant decrease in human sperm respiratory efficiency, measured polarographically, in idiophatic asthenozoospermia (Ferramosca *et al.*, 2008).

If sperm motility and its pathological aspects, including asthenozoospermia, are indeed related to mitochondrial functionality, it should be expected that mutations in the mitochondrial genome (mtDNA) may affect sperm physiology. Indeed, mtDNA encodes 13 polypeptide subunits of the mitochondrial ATP-generating pathway, along with the necessary tRNAs (Anderson *et al.*, 1981). Some subunits of the mitochondrial respiratory complexes I, III and IV are encoded by mitochondrial genome. The other components are encoded by nuclear genome. Alterations of mtDNA, such as single or multiple deletions or even modifications of its content inside mitochondria, cause a decrease in sperm motility and, consequently, an impaired fertility (Folgero *et al.*, 1993; Kao *et al.*, 1995, 2004; Spiropoulos *et al.*, 2002; Diez-Sanchez *et al.*, 2003). Furthermore, it has been proposed that some human mtDNA variants are responsible for a decreased mitochondrial OXPHOS and, consequently, for a reduced spermatozoa motility (Ruiz-Pesini *et al.*, 2004; Montiel-Sosa *et al.*, 2006). However, these experiments led to contrasting results because of specific technical and interpretational controversies. There is, however, a large consensus on the conclusion that alterations in the mitochondrial genome can compromise several sperm functions, including the motility. As this particular aspect was recently reviewed by Ramalho-Santos *et al.* (2009), this topic will not be further discussed here and readers are referred to this review for further details and references.

Sperm mitochondria functionality in humans and other mammals

It is more difficult to obtain sperm samples from humans, especially from healthy individuals, than from other mammalian species and this justifies the extensive literature regarding animal studies. Spermatozoa from bull, one of the most investigated species, are able to use the energy deriving from both glycolysis and OXPHOS for sperm motility (for a review see Storey, 2008). Succinate, pyruvate, lactate and acetate are some of the substrates employed for mitochondrial OXPHOS. Very interestingly, bull spermatozoa also utilize cellular phospholipids, as well as glycerol 3-phosphate, as energy sources for mitochondrial OXPHOS. The importance of glycerol is confirmed by the finding that glycerolphosphorylcholine diesterase catalyses the release of free glycerol in the cow oviduct. Differently from many other animal species, glucose inhibits bull sperm capacitation (Parrish *et al.*, 1989). It has been proposed that the glucose-derived lactate decreases intracellular pH, thereby inhibiting capacitation of bull spermatozoa (Galantino-Homer *et al.*, 2004). For this reason, the concentration of glucose is very low in the cow oviduct lumen (Carlson *et al.*, 1970). The fluorimetric determination of $\Delta\psi_m$ further confirmed the importance of mitochondrial OXPHOS for the motility of bull spermatozoa (Ericsson *et al.*, 1993).

The primary role of mitochondrial OXPHOS was also demonstrated in ram and boar using different approaches (for a review see Storey, 2008; Windsor, 1997). Interestingly, boar sperm mitochondria oxidize glycerol 3-phosphate (Calvin & Tubbs, 1978), as already observed in the case of bull sperm. In ram and boar, as well as in horse, dog and human, the length of sperm tail is quite short in comparison to rodents, in which the length is much longer (Cummins & Woodall, 1985). The diffusion of mitochondrial ATP, produced at the level of sperm midpiece, is undoubtedly facilitated in the case of shorter tails. The mitochondrial oxidation of glycerol 3-phosphate was also found in the case of mouse spermatozoa (Carey *et al.*, 1981).

A lot of work on sperm mitochondrial metabolism has also been carried out in rabbit, in which, for the first time, a hypotonic swelling of spermatozoa was carried out (Keyhani & Storey, 1973). Hypotonically treated rabbit spermatozoa respired very efficiently after the addition of pyruvate and malate (Keyhani & Storey, 1973). The technique of hypotonic swelling was subsequently used in other animal species, such as boar (Calvin & Tubbs, 1978), bull (Storey, 1980), mouse (Carey *et al.*, 1981), rat (Piasecka *et al.*, 2001) and human (Ferramosca *et al.*, 2008). Differently from bull, rabbit sperm mitochondria did not respire efficiently with glycerol 3-phosphate (Storey & Keyhani, 1974a). Moreover, a prominent role of glycolysis was proposed in rabbit spermatozoa on the basis of the high activities of pyruvate kinase and dynein-ATPase in sperm flagellum (Storey & Kayne, 1980).

The important role of glycolysis was also proposed in mice, in which spermatozoa remain motile also after the addition of reagents capable of uncoupling OXPHOS (Travis *et al.*, 2001). Almost the same conclusion was reached by other authors (Mukai & Okuno, 2004) who, however, noted that mice spermatozoa remained motile if pyruvate or lactate were supplied. It was recently reported that mice in which the gene encoding the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase was knocked-out have immotile spermatozoa (Miki *et al.*, 2004). The authors concluded that an active glycolysis is absolutely required in mice to support sperm motility (Miki *et al.*, 2004). The validity of this conclusion was, however, questioned by Ford (Ford, 2006) on the basis of previous results obtained using specific inhibitors of the same cytosolic enzyme. In fact, a drug-inhibited glyceraldehyde 3-phosphate dehydrogenase did not impair the motility of rat spermatozoa if these were incubated in presence of pyruvate and lactate but in the absence of glucose (Ford & Harrison, 1981). The same results were obtained in ram and boar spermatozoa (Ford & Harrison, 1985). It was proposed that when glyceraldehyde 3-phosphate dehydrogenase is blocked, no matter in which way its activity is destroyed, a non-productive use of cytosolic ATP occurs, eventually inhibiting sperm motility. This is due to the phosphorylation of glucose in the first reactions of the glycolytic pathway. However, when pyruvate or lactate are supplied yet glucose is absent, spermatozoa produce energy by mitochondrial OXPHOS. In rat, as well as in other animal species, mitochondrial functionality was assayed using a $\Delta\psi$-sensitive fluorescent dye, and this led to the discovery of a positive correlation between sperm motility and mitochondrial functionality (Gravance *et al.*, 2001). Mice spermatozoa, in which the gene encoding the sperm-specific isoform of cytochrome *c* had been knocked-out, showed a decrease in ATP content, in motility and fertilizing ability (Narisawa *et al.*, 2002).

These animals were also characterized by testicular atrophy, a phenomenon typical of ageing as a consequence of a decreased mitochondrial OXPHOS (Narisawa *et al.*, 2002). On the other hand, mice defective in mitochondrial DNA polymerase were also infertile (Trifunovic *et al.*, 2004). In addition, these animals manifested a premature onset of ageing-related phenotypes.

The role of glucose in sperm hyperactivation and/or capacitation has been largely investigated in mouse spermatozoa (Fraser & Quinn, 1981; Urner *et al.*, 2001), although it is unclear whether this hexose is required for providing extra metabolic energy through glycolysis, or for generating some other metabolic product. Glucose is necessary to initiate both the acrosome reaction and the whiplash motility associated with sperm fertilizing ability (Fraser & Quinn, 1981) and thus may have an influence on sperm protein tyrosine phosphorylation (Urner *et al.*, 2001).

Calcium and sperm mitochondria

Ca^{2+} signalling is of particular importance in sperm cells, where it regulates many processes, such as capacitation, hyperactivation, chemotaxis and acrosome reaction (Publicover *et al.*, 2007, 2008). Mitochondria play an important role in modulating intracellular Ca^{2+} homoeostasis, as they are able to function as intracellular Ca^{2+} stores (Costello *et al.*, 2009). Ca^{2+} uptake is driven by the negative membrane potential on the matrix side and is undertaken by the mitochondrial Ca^{2+} uniporter (MCU) located in the organelle's inner membrane (Kirichok *et al.*, 2004).

In somatic cells, Ca^{2+} activates mitochondrial OXPHOS stimulating several dehydrogenases of the Krebs cycle, the electron transport chain and the F_o-F_1ATP synthase (Gunter *et al.*, 1994; Hansford, 1994; Territo *et al.*, 2000). Therefore, a parallel increase in cytosolic and mitochondrial matrix Ca^{2+} concentrations coordinates mitochondrial ATP production with cellular energy demand. What happens in spermatozoa in this respect? Numerous studies have demonstrated that extracellular Ca^{2+} has a regulatory role in the control of motility in spermatozoa of several species. Extracellular Ca^{2+} strongly affects motility of intact hamster, mouse and rat sperm (Davis, 1978; Morton *et al.*, 1978) and stimulates motility of hamster, bovine and human spermatozoa (Babcock *et al.*, 1976; Lui & Meizel, 1979; Fakih *et al.*, 1986). A relationship between intracellular calcium concentration, energy metabolism, and sperm motility was found in ram mitochondria (Breitbart & Nass-Arden, 1995). In this study, sperm motility dependent on mitochondrial activity was significantly inhibited by exogenous calcium, while glycolytic-dependent motility was unaffected under these

conditions. Furthermore, in hypotonically treated rabbit epididymal spermatozoa the succinate-induced respiration was increased by 50% upon addition of Ca^{2+} in the presence of oligomycin and rotenone (Storey & Keyhani, 1974b). Demembranated spermatozoa, an excellent model for investigating sperm motility and hyperactivation by allowing direct access of reagents to the axoneme, have been used to demonstrate that Ca^{2+} acts directly on the flagellar axoneme to stimulate hyperactivation (Ho *et al.*, 2002). In this study, the authors demonstrated that in bovine sperm, which are immotile following membrane removal and disruption of the mitochondria, the addition of ATP and Ca^{2+} was able to restore motility and hyperactivation. It is, however, uncertain whether mitochondria play a role during these events. In a later study (Ho & Suarez, 2003) the same authors found that bull spermatozoa did not show an increase in NADH or in ATP production when Ca^{2+} was released from intracellular stores by thapsigargin to induce hyperactivation. This study, which is focused on the initiation of hyperactivation, suggests that the released Ca^{2+} affects the axoneme directly, without intervention of mitochondria. However, it is possible that the maintenance of hyperactivation for long periods may require a Ca^{2+}-induced activation of mitochondria (Ho & Suarez, 2003).

It is therefore clear that multiple relationships exist between mitochondria and Ca^{2+} in sperm cells. On the one hand, mitochondria accumulate and release Ca^{2+} and in this way they can indirectly modulate some Ca^{2+}-mediated intracellular effects. On the other hand, Ca^{2+} accumulated in the matrix may stimulate multiple energy-producing reactions thereby increasing intramitochondrial ATP levels. High ATP, in its turn, can be used as a fuel for sperm activated or hyperactivated motility.

Reactive oxygen species and sperm mitochondria

Reactive oxygen species (ROS) are a group of free radicals that in high concentration have negative influence on sperm quality and function. Sperm cells, as well as the seminal plasma, possess several antioxidant factors, which are generally able to efficiently counteract this oxidative stress. An unbalance between oxidative stress and ROS scavenging may lead to male infertility. Mitochondria are the major ROS generator, as they convert 0.2–2% of the oxygen taken up by the cells to ROS (Harper *et al.*, 2004; Murphy, 2009). In spermatozoa, mitochondrial Complex I and Complex III are the major sites for ROS production (Koppers *et al.*, 2008). In somatic mitochondria additional sources of ROS are Complex II (Zhang *et al.*, 1998), glycerol 3-phosphate dehydrogenase (Drahota *et al.*, 2002) or a fraction of p66[Shc], a mitochondrial protein localized in the intermembrane space that produces hydrogen peroxide by accepting electrons from reduced cytochrome *c* (Giorgio *et al.*, 2005).

An important area of controversy is to which side of the inner mitochondrial membrane either Complex I or Complex III releases superoxide (either to the mitochondrial matrix side or the cytoplasmic one). Muller *et al.* (2004) demonstrated that Complex I-dependent superoxide is exclusively released into the matrix, while Complex III can release superoxide to both sides of the inner mitochondrial membrane. In this way, mtDNA which is localized in the mitochondrial matrix, is exposed to oxidative damage by ROS. mtDNA is highly susceptible to oxidative damage because of its high turnover rate, lack of protection by histones and limited capacity of mitochondria to repair DNA damage. As the molecules of sperm mtDNA are very few (100–1000) as compared with mtDNA content in somatic cells (10^2–10^4 copies), mtDNA mutations in spermatozoa manifest early as hypospermatogenesis and later as motility defects (Kumar *et al.*, 2009; Venkatesh *et al.*, 2009). Therefore, mtDNA alterations caused by ROS have profound adverse effects on sperm motility and, consequently, on fertility potential (Folgero *et al.*, 1993; Kao *et al.*, 1995, 2004; Spiropoulos *et al.*, 2002; Diez-Sanchez *et al.*, 2003). In addition, sperm lipoperoxidation damage induced by oxidative stress may be another cause of male infertility (Storey, 2008; Ramalho-Santos *et al.*, 2009).

On the other hand, it has been suggested that small amounts of mitochondrial ROS are necessary for spermatozoa to acquire fertilizing capabilities (Griveau & Le Lannou, 1997). Co-incubation of spermatozoa with low concentrations of hydrogen peroxide stimulates sperm capacitation, hyperactivation, acrosome reaction and oocyte fusion (Griveau *et al.*, 1994; Aitken, 1995; Kodama *et al.*, 1996). ROS such as nitric oxide (NO) and the superoxide anion have also shown to promote capacitation and acrosome reaction (Griveau *et al.*, 1995).

Reactive oxygen species can therefore show beneficial or detrimental effects on sperm vitality and functions in dependence on their nature and concentration (de Lamirande & Gagnon, 1995). A malfunctioning of mitochondria, or a deficit of antioxidant protection, can negatively affect sperm fertility without a direct interference with sperm motility.

Concluding remarks

The survey of the literature previously reported reveals that a lot of work has actually been performed but that many aspects of sperm mitochondrial bioenergetics require further investigation. In our opinion, the metabolism of sperm mitochondria is often regarded as quite similar to that of somatic cells but, in some cases, this

can be the cause of misleading interpretations. A better strategy should imply the design of a novel framework for sperm mitochondrial bioenergetics, keeping in mind that spermatozoa are terminal cells that must survive in the female reproductive tract. Therefore, in the following paragraphs we summarize some hints for future investigations in this field.

Sperm mitochondria contain at least three distinct shuttles (lactate/pyruvate, aspartate/malate and glycerol 3-phosphate) for the transport of reducing equivalents into the matrix. However, many questions are still unanswered in this respect. First of all, do all three shuttles operate in human sperm mitochondria? Then, if so, do they operate with the same efficiency or does their importance change according to the metabolic state of the cells? Furthermore, what are the characteristics of the putative lactate carrier of human sperm mitochondria? Appropriate experiments should be designed to clarify these points using human sperm samples in which a direct assay of metabolite flux across the inner mitochondrial membrane is carried out.

In spermatozoa, the metabolism of lactate appears particularly intriguing. Although this molecule in somatic cells is simply viewed as a final product of glycolysis, especially in anaerobic conditions, in spermatozoa it is probably involved in multiple reactions. Unfortunately, most of them, occurring inside and outside mitochondria, are only partially known. It is surprising that LDH-X inside mitochondria allows the reduction of pyruvate to lactate hence decreasing the concentration of NADH, which is instead normally used by the respiratory chain for energy production. Does this reaction really compete with mitochondrial OXPHOS and, if so, why and when does this happen in human spermatozoa? It would also be interesting to calculate the ΔG for the reactions catalysed by LDH-X inside and outside sperm mitochondria by taking into consideration the actual concentrations of both lactate and pyruvate (and of other reagents such as H^+). Indeed, these reactions could either produce or consume NADH thereby causing gross variations in the reducing equivalent availability for the mitochondrial respiratory chain. In other words, it would be interesting to understand the logic of these dehydrogenations in the context of sperm bioenergetics.

Furthermore, although bull sperm mitochondria are capable of obtaining energy from cellular phospholipids oxidation, this is still unknown in the case of human samples. This phenomenon is not seen, as far as we know, in the case of other cellular types, which normally require the integrity of their structural components. Spermatozoa, on the contrary, are terminal cells that can use all molecular components, even the structural ones, to produce energy for the final event of egg fertilization.

Moreover, the role of carnitine in human spermatozoa merits further investigation. In fact, at first it should be clarified whether sperm mitochondria of some mammals are really capable of internalizing acyl-CoA. Human sperm mitochondria, on the contrary, transport into the matrix acyl-carnitine in exchange for free carnitine. This flux across the inner membrane is catalysed by the human sperm CAC but this leads to some interesting questions: what is the carrier responsible for acyl-CoA transport in other mammalian species? Does it exist also in human sperm mitochondria? Secondly, an important pool of acetyl-carnitine is present in human and mammalian sperm mitochondria. Yet, what is the function of this pool and what is its relationship with other metabolic pathways operating inside sperm mitochondria?

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