Zygote

cambridge.org/zyg

Review

Cite this article: Losano JDA *et al* (2018) Spermatic mitochondria: role in oxidative homeostasis, sperm function and possible tools for their assessment. *Zygote* **26**: 251–260. doi:10.1017/S0967199418000242

Received: 12 December 2017
Revised: 22 May 2018
Accepted: 22 June 2018
First published online: 18 September 2018

Keywords:
Glycolysis; Mitochondrion; Oxidative phosphorylation; Reactive Oxygen Species; Sperm

Author for correspondence:
Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Orlando Marques de Paiva, 87-05508-270, São Paulo, Brazil. Tel: +55 11 30911423. Fax: +55 11 30911437. E-mail: jdalosano@usp.br

© Cambridge University Press 2018.

CAMBRIDGE UNIVERSITY PRESS

Spermatic mitochondria: role in oxidative homeostasis, sperm function and possible tools for their assessment

João Diego de Agostini Losano, Daniel de Souza Ramos Angrimani, Roberta Ferreira Leite, Bárbara do Carmo Simões da Silva, Valquíria Hyppolito Barnabe and Marcilio Nichi

Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, University of São Paulo, Brazil.

Summary

Despite sperm mitochondrial relevance to the fertilization capacity, the processes involved in the production of ATP and functional dynamics of sperm mitochondria are not fully understood. One of these processes is the paradox involved between function and formation of reactive oxygen species performed by the organelle. Therefore, this review aimed to provide data on the role of sperm mitochondria in oxidative homeostasis and functionality as well the tools to assess sperm mitochondrial function.

Introduction

Nuclear power, including the production of low-carbon electricity, is responsible at this time for almost one-third of global total energy generation. According to the International Atomic Energy Agency (IAEA), the main reason for this use is the low investment cost relative to the amount of energy produced and the fact that nuclear power plants produce virtually no greenhouse gas emissions or air pollutants during their operation, and very low emissions over their entire life cycle. Therefore, due to these benefits, there are now 447 nuclear power reactors in operation in 30 countries and another 58 reactors are under construction, based on IAEA information. However, the use of nuclear energy remains a cause of concern around the world, due to the devastating effects of accidents caused by core damage in nuclear plants. This concern is based on the long-term consequences of accidents, such as the accidents at Chernobyl (1986) and Fukushima-Daiichi (2011).

As in nuclear power plants, mitochondria exhibit high energy production capacities. However, in situations in which the structure of this organelle is compromised, the potential to release extremely toxic products is also worrying. Such toxic substances may lead to damage of the surrounding cells and other tissues. In fact, several studies have linked mitochondrial dysfunction to some pathological conditions such as neurodegenerative diseases (Lin & Beal, 2006), type 2 diabetes (Lowell & Shulman, 2005) and neoplasia (Modica-Napolitano & Singh, 2004).

In regards to the spermatozoa, several studies related mitochondria to be the main source of cell energy, playing an important role in cellular homeostasis and sperm motility (Travis *et al.*, 1998; St John, 2002). However, for some species, evidence suggests that glycolysis may be also an important source of ATP production for sperm motility, superior to oxidative phosphorylation (Mukai and Okuno, 2004; Ford, 2006; Nascimento *et al.*, 2008).

Despite the importance of mitochondria to sperm metabolism, during oxidative phosphorylation, metabolites called reactive oxygen species (ROS) are produced and are a trigger for several reproductive physiological mechanisms (de Lamirande *et al.*, 1997). Nevertheless, an unbalance between ROS production and mechanisms aiming to avoid their powerful oxidative potential (i.e. antioxidants), may be extremely harmful to the spermatozoa (Halliwell, 1999; Nichi *et al.*, 2007a).

In this context, mitochondria are highlighted as source of pro-oxidative factors that are crucial in the disruption of oxidative homeostasis (Agarwal *et al.*, 2014). Several studies have demonstrated correlations between impaired mitochondrial activity, oxidative stress and sperm DNA fragmentation, indicating a close relationship between these variables on sperm damage (Barros, 2007; Nichi *et al.*, 2007b; Blumer *et al.*, 2012).

Since the accident at Chernobyl, several safety improvements have been adopted and, after the Fukushima accidents, new generations of more safe designs for nuclear power stations have been developed. The main concern of nuclear energy specialists and the community, in general, is the approaches made to prevent the destruction and long-term consequences

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

caused by an eventual nuclear disaster. If possible, the deactivation of power plants prior to predictable stressful events would probably avoid most damage. Similarly, mitochondrial therapy is applied in situations in which organelle dysfunction occurs (i.e., sperm cryopreservation) (O'Connell *et al.*, 2002; Sariozkan *et al.*, 2009; Thomson *et al.*, 2009), and aimed to improve sperm viability by prevention of pro-oxidative factors release. Actually, some studies have suggested that, for certain cell types, uncouplers of oxidative phosphorylation are capable of reducing oxidative stress (Vincent *et al.*, 2004; Mailloux & Harper, 2011).

This review aimed to compile available data on the role of mitochondria in oxidative homeostasis and sperm functionality as well as suggesting some tools to assess the sperm mitochondrial function.

The mitochondrial paradox: physiological and pathological role on spermatozoa

According to the endosymbiotic theory, millions of years ago the mitochondrion was a prokaryotic unicellular organism. Formerly a free-living bacterium, the mitochondrion was capable of metabolizing oxygen in an environment rich in carbon dioxide. After penetrating a host eukaryotic cell that was incapable of metabolizing oxygen, a symbiotic relationship was established, later evolving into a more complex organism capable of producing energy more efficiently than the previously available glycolysis pathways (Margulis, 1970; Cummins, 1998). In fact, aerobic metabolism is highly dependent on mitochondrial functionality. The aerobic respiration is then, a consequence of the mitochondrial demand for oxygen which, by means of oxidative phosphorylation, is capable of producing approximately 90% of cellular energy (Saraste, 1999; Copeland, 2002).

Role of mitochondria in ATP production and sperm physiology

Studies have demonstrated the main role of mitochondria on sperm functionality, referring to this organelle as the main source of ATP for cellular homeostasis and motility (Travis *et al.*, 1998; St John, 2002). However, its role in sperm metabolism has been a matter of debate. Mukai & Okuno (2004), when inhibiting sperm mitochondrial activity in mice concomitantly to glycolytic pathway supplementation, observed that ATP production and flagella beat remained unaltered. However, when glycolysis was inhibited and oxidative phosphorylation was stimulated, a drastically reduction in flagella beat and ATP production occurred. This finding suggested that glycolysis is more relevant than oxidative phosphorylation in the energetic metabolism of murine sperm. In a recent study conducted by our group, we observed similar results in bovine epididymal spermatozoa subjected to mitochondrial uncoupling and glycolysis stimulation (Losano *et al.*, 2017a; Fig. 1). In addition, Nascimento *et al.* (2008) observed similar results in human sperm. These authors suggested that, despite the important contribution of oxidative phosphorylation for ATP production, glycolysis is the primary source of energy in human sperm. Conversely, other studies in humans have described the opposite effect when sperm samples are incubated with inhibitors of the enzymatic electron transport complexes, with a decrease in sperm motility (Ruiz-Pesini *et al.*, 2000; John *et al.*, 2005). Furthermore, we verified that ovine sperm undergoing mitochondrial depolarization that did not alter their total motility. Spermatic kinetic patterns were affected, suggesting that mitochondria are very important in maintaining the quality of ovine spermatozoa movement (Losano *et al.*, 2017b; Fig. 2).

Mitochondria are essential to sperm functionality due to the relationship between their functional and fertilizing capacities (Marchetti *et al.*, 2002, 2004; Gallon *et al.*, 2006; St John *et al.*, 2006). Nonetheless, it is still not clear how mitochondria can contribute to the energy capacity of sperm. The organelle has distinct contributions to sperm metabolism, dependent on experimental conditions and animal species (Storey, 2008; Amaral *et al.*, 2013).

The importance of the glycolytic pathway on ATP generation and on sperm function, has been described previously (Mukai & Okuno, 2004). Lardy and colleagues (1945) first showed that mitochondrial inhibition leads to asthenospermia. However, with glucose supplementation to the samples, sperm motility was reacquired. In addition, White & Wales (1961) observed that ovine sperm maintain their motility through two parallel mechanisms of energy generation, i.e. glycolysis and oxidative phosphorylation. Moreover, Krzyzosiak and colleagues (1999) also observed that bovine sperm were capable of maintaining similar motility patterns in both aerobic and anaerobic conditions, assuming that glycol sable substrates are available. Furthermore, previous studies have suggested that ATP molecules supplied by oxidative phosphorylation in the sperm midpiece are not efficiently diffused to the more distal regions of the tail, indicating that glycolysis would probably play a key role in flagella beat in this region (Nevo & Rikmenspoel, 1970; Turner, 2003).

Role of calcium on mitochondrial function

A hypothesis on the main regulatory mechanisms of oxidative phosphorylation considers ADP and inorganic phosphate as feedback substrates for ATP synthesis through several cellular kinases. Therefore, an interesting analogy can be employed with the economic model of supply and demand, with ATP as the unit price for cellular energy. Evidence to support this theory showed that mitochondria isolated in suspension increased their ATP production when ADP and inorganic phosphate was supplemented in the presence of oxygen. Despite the well known 'economic model of equilibrium', recent studies have shown that ATP synthesis rate is not strictly controlled by such mechanisms (Gunter *et al.*, 2004).

Mitochondrial calcium ($[Ca^{2+}]_m$) has been referred to as the central regulator of oxidative phosphorylation, acting as the primary metabolic mediator for NADH production and activity controller of the enzymatic complexes pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (McCormack *et al.*, 1990; McCormack & Denton, 1993). The $[Ca^{2+}]_m$ is also directly involved in ATP production, playing an important role in ADP phosphorylation through the enzyme ATP synthase (Territo *et al.*, 2001). Moreover, mitochondrial calcium also participates in the apoptotic mechanism of somatic cells, triggering the release of pro-apoptotic agents by the mitochondria (Szalai *et al.*, 1999).

If $[Ca^{2+}]_m$ action on physiological processes of somatic cells is established, the precise role of this ion in sperm mitochondria is still under debate (Amaral *et al.*, 2013). In a proteomic approach, studies identified sperm mitochondrial calcium uniporter (MCU) proteins that are responsible for controlling mitochondria calcium signalling, metabolism and cellular survival. However, sperm mitochondrial calcium concentration is seemingly unaltered by mitochondrial uncoupling (Machado-Oliveira *et al.*, 2008; Wang *et al.*, 2013). Additionally, in bulls, mitochondrial activity on hyperactivated sperm appears to be unregulated by calcium release. In this context, further studies are vital to

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

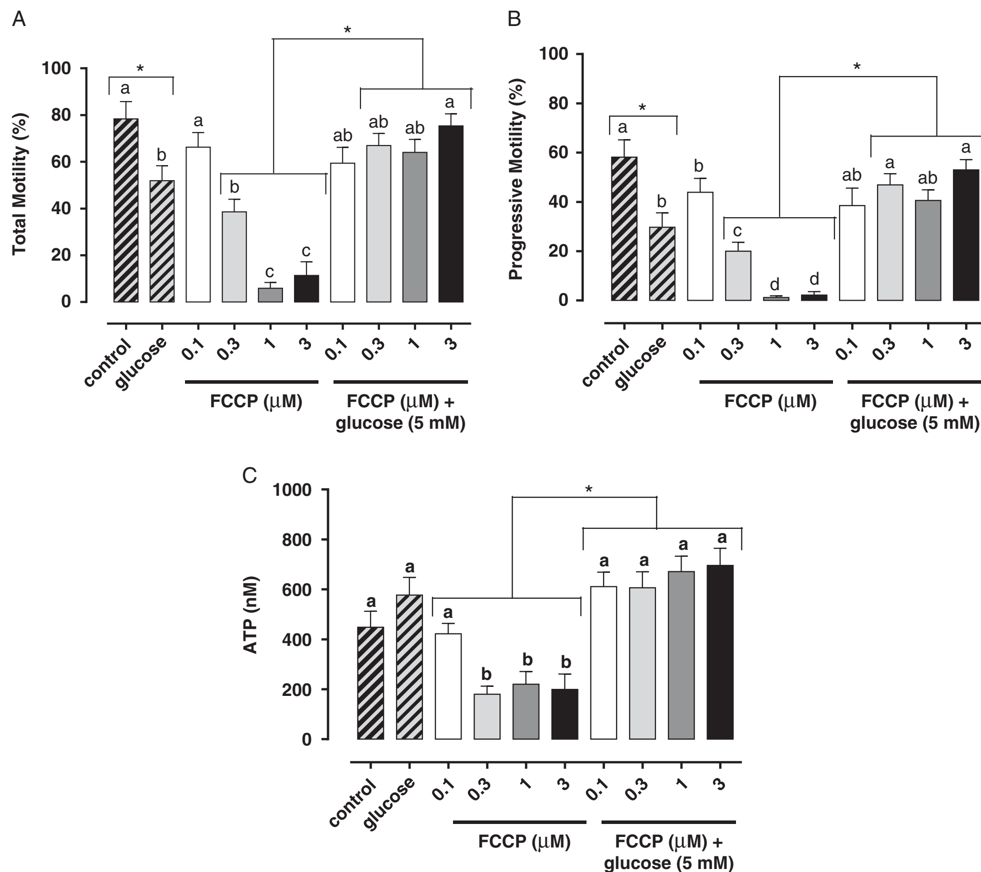

Figure 1. In this study, we verified that the stimulated glycolytic pathway (glucose 5 mM) is able to maintaining total (*A*) and progressive (*B*) motilities and ATP levels (*C*) of bovine epididymal spermatozoa subjected to mitochondrial uncoupling [carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP); 0.1, 0.3, 1 and 3 µM] (Losano *et al.*, 2017a). [a,b,c,d]Different letters on the bars indicate significant differences between treatments ($P < 0.05$).

establish the real function of calcium in mitochondrial physiology, the reference values for $[Ca^{2+}]_m$, and to correlate such values with sperm function (Irvine and Aitken, 1986; Ramalho-Santos *et al.*, 2009; Amaral *et al.*, 2013).

Reactive oxygen species and the spermatozoa

During aerobic cell metabolism, ROS are formed. This event occurs firstly because the mitochondrial environment is rich in oxygen and electrons, and almost all of these electrons participate in the reduction of oxygen directly to water, the final product of oxidative phosphorylation. Physiologically, some of these electrons escape from the oxidative phosphorylation enzymatic complex and bind to molecular oxygen, leading to first ROS, the superoxide anion, generation. From this primary product, a redox reaction cascade occurs leading to the formation of other reactive oxygen species, such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) respectively.

ROS produced are involved in many physiological triggers such as sperm hyperactivation (de Lamirande & Cagnon, 1993), sperm capacitation (Aitken *et al.*, 2004), acrosome reaction (de Lamirande *et al.*, 1998), and interaction between spermatozoa and the zona pellucida (Aitken *et al.*, 1995). While ROS are formed by other mechanisms, such as glycolysis, mitochondria are the main ROS source with approximately 2% of consumed oxygen being converted to superoxide anions (Koppers *et al.*, 2008).

Some enzymatic and non-enzymatic antioxidants act synergistically to prevent ROS accumulation, in which each of these metabolites is inactivated by specific antioxidants. Superoxide dismutase (SOD) is considered the primary line of antioxidant defence acting through dismutation of two molecules of superoxide anion (O_2^-) forming an oxygen molecule and a hydrogen peroxide molecule (H_2O_2; Fig. 3) (Alvarez *et al.*, 1987). Hydrogen peroxide can be destroyed by two antioxidants independent systems, the enzyme catalase and the glutathione peroxidase/reductase systems (Fig. 3; Nordberg & Arnér, 2001). If these two systems fail, the H_2O_2 will react with an Fe^{2+} or Cu^+ molecule (called the Fenton reaction) and will produce the hydroxyl radical (OH^-, Fig. 3). This ROS is considered the most reactive in biological systems, and can be destroyed by non-enzymatic antioxidants such as ascorbic acid and α-tocopherol (Fig. 3; Halliwell & Gutteridge, 1985).

Mitochondrial dysfunctions and spermatozoa

Despite the physiological function, any imbalance in ROS production and antioxidant mechanisms can lead to oxidative stress, which may be lethal for sperm cells (Fig. 4; de Lamirande *et al.*, 1997; Agarwal *et al.*, 2004). Sperm is particularly susceptible to oxidative stress due to a limited amount of cytoplasm and consequently low antioxidant activity and also a high quantity of polyunsaturated fatty acids which is easily oxidized. Thus, oxidative stress may cause damage to different sperm structures such as in plasma and acrosomal membranes, mitochondria and sperm DNA. Spermatozoa cannot restore damages caused by oxidative stress due to deficiency of cytoplasmic repair enzymes (Vernet *et al.*, 2004; Nichi *et al.*, 2007b; Agarwal *et al.*, 2014).

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

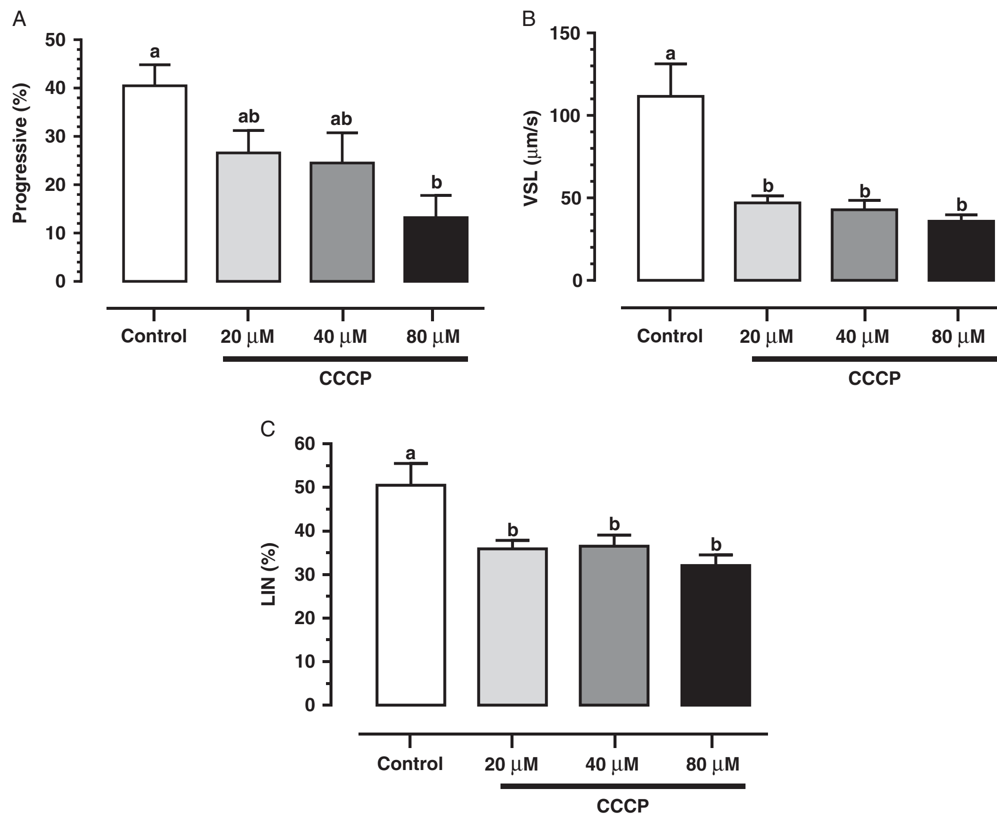

Figure 2. We verified that mitochondrial uncoupling [carbonyl cyanide 3 chlorophenylhydrazone (CCCP); 20, 40 and 80 μM] impairs ovine sperm kinetic patterns such as progressive motility (*A*), straight-line velocity (VSL; *B*) and linearity (LIN; *C*), indicating an essential role of mitochondria to sperm quality movement related to progressivity (Losano *et al.*, 2017b). [a,b]Different letters on the bars indicate significant differences between treatments ($P < 0.05$).

As mitochondria are the major source of pro-oxidative agents, it is suggested therefore that dysfunction in this organelle would have a fundamental role in the oxidative imbalance affecting sperm function (Agarwal *et al.*, 2014). Wang and colleagues (2003) identified low mitochondrial membrane potential and high ROS production in sperm from infertile patients, probably as a consequence of such mitochondrial injury, suggesting that mitochondrial function can be a marker of male fertility. In fact, other researchers have observed changes in mitochondrial function in sperm derived from infertile men (Troiano *et al.*, 1998; Gallon *et al.*, 2006). However, sperm samples with high mitochondrial membrane potential have been identified in fertile patients (Kasai *et al.*, 2002; Marchetti *et al.*, 2002).

Studies performed in different species have shown a negative correlation between both oxidative stress and high mitochondrial activity. The occurrence of this stress and the sperm DNA integrity indicated that these variables are linked and lead a single pathogenic mechanism (Barros, 2007; Nichi *et al.*, 2007b; Blumer *et al.*, 2012). Correlation was also found between variables in spermatic oxidative stress and lower blastocyst rates, as rates of blastomeres increased with DNA damage, confirming the negative effect of seminal oxidative stress in *in vitro* embryonic development (Simões *et al.*, 2013).

Mitochondrial disorders have multifactorial origins, and some mechanisms have not been totally elucidated (Amaral *et al.*, 2013). These changes can be triggered even in the testis during spermatogenesis, for example if the testicular thermoregulatory mechanism is inefficient. Only 50% of the blood supply reaches the testes through the testicular artery, therefore male gonads are subjected to near hypoxic environments (Meijer & Fentener Van Vlissingen, 1993). Testis metabolism increased as consequence of pathological conditions that raised testicular temperature and were not compensated by increase in blood flow, causing a testicular hypoxic condition (Paul *et al.*, 2009). Beyond these conditions and at the beginning of oxygenation, there is an increase in ROS production that leads to oxidative stress. This mechanism is known as ischaemia-reperfusion injury (Nichi *et al.*, 2006; Reyes *et al.*, 2012). Increase in ROS production in this condition is related to mitochondrial dysfunction and the subsequent activation of enzymes that play a role in a ROS generated systems, such as xanthine oxidase (XO). These changes in the mitochondria are related to lack of O_2 during ischaemia, which leads to ATP depletion and consequently mitochondrial injury. Moreover, the increase in testicular temperature promotes an influx of calcium and is also related to changes in this organelle (Dorweiler *et al.*, 2007; Reyes *et al.*, 2012).

Sperm cryopreservation is a key process in assisted reproduction techniques (Hammerstedt *et al.*, 1990; Zapzalka *et al.*, 1999; Holt, 2000). However this technique promotes a decrease in sperm quality, and also in mitochondrial damage during cryopreservation due to excessive production of pro-oxidative factors that, ultimately, cause post-thaw sperm damage and decrease in motility (O'Connell *et al.*, 2002; Sariozkan *et al.*, 2009; Thomson *et al.*, 2009). Additionally, the process promotes a reduction in antioxidant capacity after sperm cryopreservation, a further factor that also predisposes these cells to oxidative stress (Bilodeau *et al.*, 2000).

Consequently, several studies have used antioxidant treatment in sperm samples submitted to cryopreservation, aimed at preventing oxidative stress caused by mitochondrial injuries (Askari *et al.*, 1994; Bilodeau *et al.*, 2001; Fernández-Santos *et al.*, 2007;

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

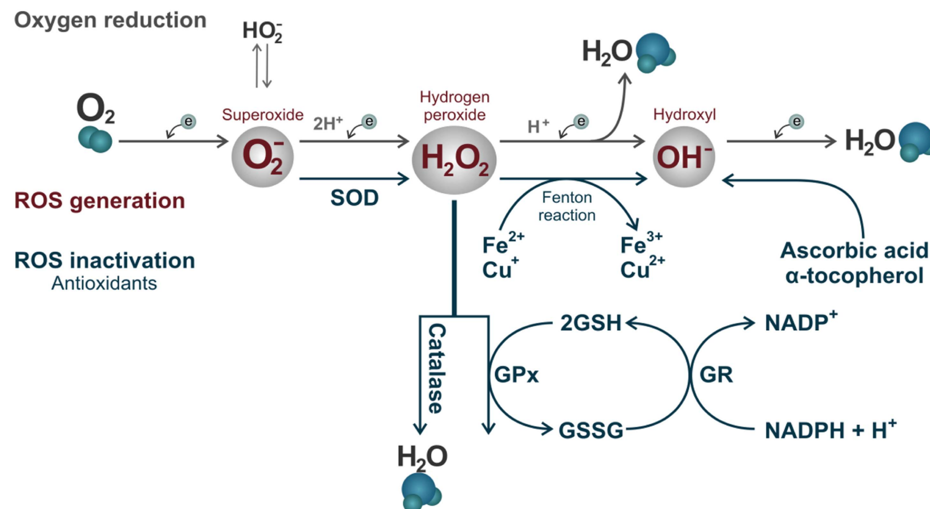

Figure 3. Reactive oxygen species formed by the oxy-reduction process from O_2 to H_2O and their respective inactivation antioxidant systems. The enzyme superoxide dismutase (SOD) acting through dismutation of two molecules of superoxide anion (O_2^-) forming an oxygen molecule and a hydrogen peroxide molecule. Hydrogen peroxide (H_2O_2) can be destroyed by two antioxidants independent systems, the enzyme catalase and glutathione peroxidase (GPx)/glutathione reductase (GR) system, with the participation of oxidized (GSSG) and reduced (GSH) glutathione. If these two systems fail, H_2O_2 will react with an iron (Fe^{2+}) or (Cu^+) molecule (Fenton reaction) and will form the hydroxyl radical (OH^-). This ROS can be destroyed by non-enzymatic antioxidants such as ascorbic acid and α-tocopherol.

Taylor *et al.*, 2009). However, the use of a specific mitochondrial shield during cryopreservation also appeared as an option, aimed at improving post-thaw sperm quality (Schober *et al.*, 2007). A possible alternative would be to reduce mitochondrial activity, which can be induced by uncouplers of oxidative phosphorylation during the cryopreservation process, thus preventing ROS accumulation caused by any mitochondrial dysfunction that can occur during this process. Some uncoupler activities have been identified in the physiological processes of somatic cells, acting even in oxidative stress reduction (Vincent *et al.*, 2004; Brand & Esteves, 2005).

Inhibitors and uncouplers of oxidative phosphorylation: action mechanisms and their possible applications

Inhibitors and uncouplers of oxidative phosphorylation are important in the study of mitochondrial physiology, and have been widely used in pharmacology as many chemical compounds can inhibit the specific processes of oxidative phosphorylation. Therefore, it is possible to observe their role by preventing a single process without inhibiting other mechanisms (Nelson & Cox, 2008).

Inhibitors can act towards complex electron carriers and also in mitochondrial channels. Rotenone (e.g. insecticide class), can block the transfer of electrons from complex I to ubiquinone, inhibiting the overall process of oxidative phosphorylation (Sherer *et al.*, 2003). Conversely, antimycin A, an antibiotic produced by the *Streptomyces* fungus, blocks the transport of electrons from complex III to complex IV (Slater, 1973). Cyanide inhibits the electron transport complex IV to oxygen. Furthermore, it is possible to directly inhibit ATP synthesis with oligomycin, which is widely used in this process. This compound acts on the enzyme ATP synthase by blocking the flow of protons through the F0 subunit of this enzyme to the mitochondrial matrix and, consequently, preventing ATP synthesis (Penefsky, 1985). As well as enzyme complex inhibitors, there are also calcium channel blockers such as RU360, Na^+/Ca^{2+} pump inhibitors or CGP 37157 (García-Rivas *et al.*, 2006; Thu *et al.*, 2006).

In addition to these inhibitors, uncouplers of oxidative phosphorylation were widely used not only as a tool to study cell physiology, but also as a possible therapeutic application (Kasianowicz *et al.*, 1984). ATP synthesis occurs through coupling of two reactions, electron transport and phosphorylation, as a result of a proton gradient this class of substances uncouples these two reactions, preventing or decreasing ATP synthesis. However, electron flow activity across the mitochondrial complexes is not inhibited, and even could be increased (Terada, 1990). Most of these molecules are hydrophobic and have protonophore activity, depolarization of mitochondrial membranes allows protons to return to the mitochondrial matrix and dissipate the mitochondrial membrane potential and pH difference, inhibiting the driving proton force, essential for ATP synthesis (Chen, 1988; Terada, 1990). Uncoupling proteins have been identified in some cells and are related to some physiological roles such as in adaptive thermogenesis in adipose tissue.

Moreover, these proteins have been identified in researches related to obesity, diabetes, neurodegenerative disease and ageing in humans (Brand and Esteves, 2005). These studies emerged as previous researches found out that mitochondrial uncouplers can control mitochondria ROS production and, therefore, prevent oxidative stress, which is related to these diseases. Therefore, the use of these proteins in cell therapy for the treatment of these pathologies is suggested (Brand & Esteves, 2005; Lowell & Shulman, 2005; Lin & Beal, 2006; Mailloux & Harper, 2011). Decrease in ROS production promoted by uncouplers is due to an increase in the respiratory rate followed by a decrease in mitochondria intermediate reduced states, capable of donating single electrons to oxygen, thereby preventing the generation of superoxide anions.

The uncoupled process has been applied in an energy study of spermatozoa (Mukai & Okuno, 2004), however there is still no evidence that these compounds can control ROS production by sperm mitochondria. However, the use of these substances may bring interesting results for the prevention of oxidative stress in seminal samples in front of possible mitochondrial dysfunction. Thus, the application of this treatment can be attractive, especially for use in reproductive biotechnologies due to the highest susceptibility to oxidative stress.

Tools for assessing sperm mitochondrial function

Sperm mitochondria can be involved in both physiological as pathological processes, therefore the importance of assessing the

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

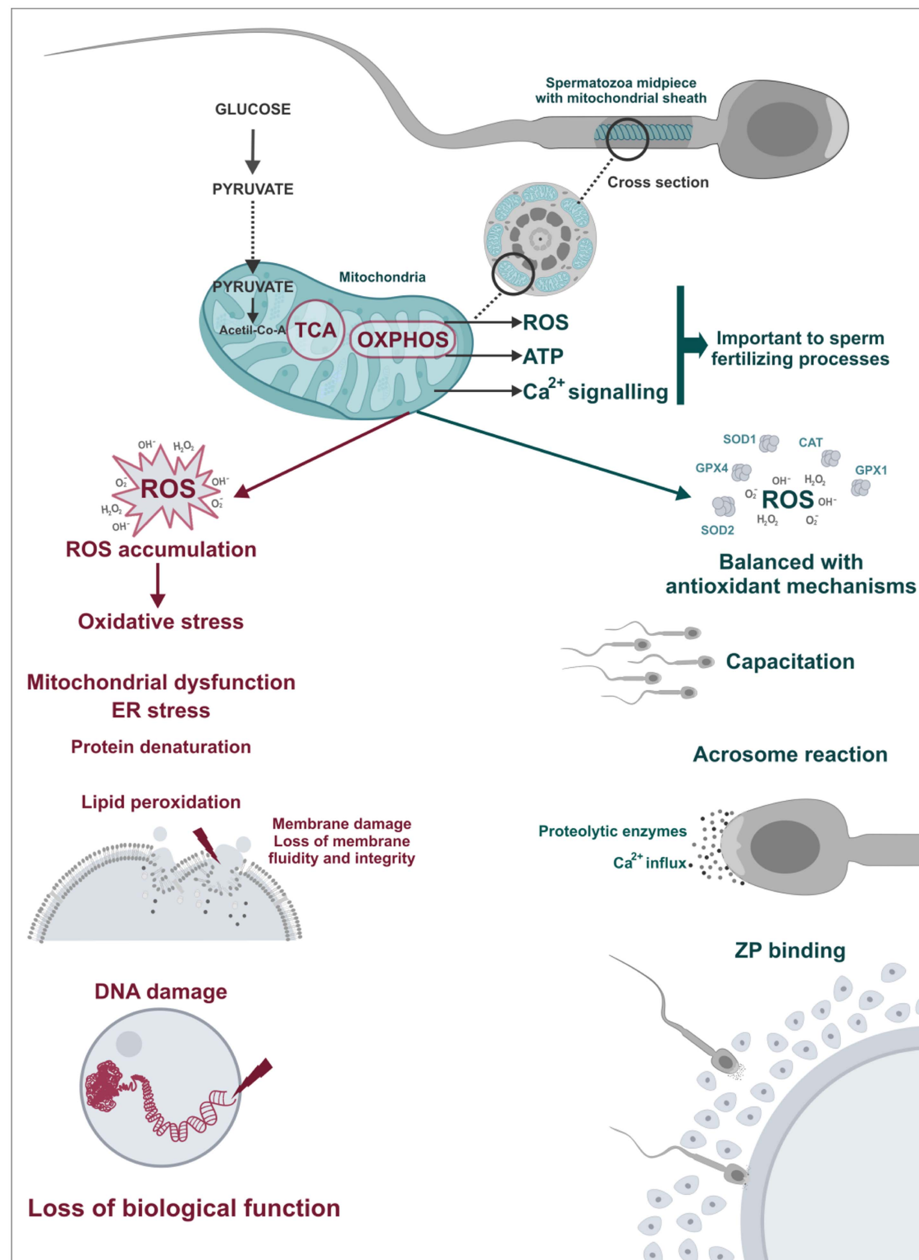

Figure 4. Physiological and pathological role of sperm mitochondria. ROS play a important role in sperm physiology acting as triggers of fertilization processes such as hyperactivation, acrosome reaction and spermatozoa–oocyte binding. However, in cases of mitochondrial dysfunctions, there is an imbalance between ROS production and antioxidant capacity, the oxidative stress. In this case, ROS cause damage to sperm structures including lipid peroxidation of the plasma membrane and DNA damage leading to loss of biological function of spermatozoa.

functionality of this organelle is evident. The use of tools to evaluate sperm mitochondrial function associated with other sperm analysis can be applied for the prediction of fertilizing capacity (Troiano *et al.*, 1998; Kasai *et al.*, 2002; Aitken, 2006). In this context, sperm mitochondria have been studied for some decades (Christen *et al.*, 1983; Hrudka, 1987; Graham *et al.*, 1990) Thus, several tools have been developed for assessment of mitochondrial function (Table 1).

Mitochondria activity evaluation aims to infer the efficiency of electron transport between enzymatic complexes and also in the redox processes involved in oxidative phosphorylation. In classic research, Hrudka (1987) developed a cytochemical technique to evaluate mitochondrial activity. This cytochemical assay is based on the oxidation of 3′3-diaminobenzidine (DAB) by cytochrome*c*, an enzyme involved in electron transport between the enzymatic complexes. Subsequently, some fluorescent probes such as H2-CMXros and CMXros, were developed with the same purpose and commercially sold as Mito Tracker Red® (Poot *et al.*, 1996; Wojcik *et al.*, 2000; Celeghini *et al.*, 2007).

Fluorescent probes, such as JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; Garner *et al.*, 1997), Mito Tracker Green FM® (Gillan *et al.*, 2005) and Rhodamine 123® (Graham *et al.*, 1990), were also developed to assess mitochondrial membrane potential. These probes diffuse freely through the plasma membrane to the cell cytosol and accumulate electrophoretically in the mitochondrial matrix, determined by proton motive force and acting in accordance with mitochondria ability to pump protons from the matrix to the intermembrane area (Chen, 1988; Garner *et al.*, 1997; Piccoli *et al.*, 2006).

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

Table 1. Available tools for assessing sperm mitochondrial functionality (mitochondrial activity, mitochondrial membrane potential and calcium levels assessments)

Technique	Assay/Probe/kit	Application/Procedure
Mitochondrial activity evaluation		
Cytochemical technique	DAB; 3′,3-diaminobenzidine	Oxidation of DAB by cytochrome *c*. Analysis by optical microscopy (Herzog & Fahimi, 1973; Pariz & Hallak, 2016)
Fluorescent probes	H2-CMXros; 8-(4′-chloromethyl) pheny1–2,3,5,6,11,12,14,15-octahydro-1H,4H,1OH,13H-diquinolizino-8H-xanthene	Stains mitochondria in live cells. The extent of dye uptake reflects the redox potential across the mitochondrial membrane. Analysis can be made by fluorescence microscopy and flow cytometry (Rasola & Geuna, 2001; Celeghini *et al.*, 2007)
	CMXros; 8-(4′-chloromethyl) pheny1–2,3,5,6,11,12,14,15-octahydro-1H,4H,1OH,13H-diquinolizino-8H-xanthylium chloride	Stains mitochondria in live cells. The extent of dye uptake reflects the redox potential across the mitochondrial membrane. Analysis can be made by fluorescence microscopy and flow cytometry (Rasola & Geuna, 2001; Celeghini *et al.*, 2007)
	Mito Tracker Red®	Stains mitochondria in live cells and its accumulation is dependent upon membrane potential. Analysis can be made by fluorescence microscopy and flow cytometry (Rasola & Geuna, 2001; Hallap *et al.*, 2005)
Mitochondrial membrane potential evaluation		
Fluorescent probes	JC-1; 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide	A cationic carbocyanine dye that accumulates in mitochondria. At higher concentrations, the dye forms J-aggregates that exhibit a broad excitation spectrum. Analysis can be made by fluorescence microscopy and flow cytometry (Smiley *et al.*, 1991; Hu *et al.*, 2017)
	Rhodamine 123®	A cell-permeant, cationic, green-fluorescent dye that is readily sequestered by active mitochondria without cytotoxic effects. Analysis can be made by fluorescence microscopy (Forster *et al.*, 2012; Celeghini *et al.*, 2007)
	Mito Tracker Green FM®	MitoTracker Green FM is green-fluorescent mitochondrial stain which localizes to mitochondria regardless of mitochondrial membrane potential. The dye stain live cells but is not well-retained after aldehyde fixation. Analysis can be made by fluorescence microscopy and flow cytometry (Chazotte, 2011; Celeghini *et al.*, 2007)
Mitochondrial calcium levels evaluation		
Fluorescent probes	Quin-2 AM; 2-[(2-amino-5-methylphenoxy)methyl]-6-methoxy-8-aminoquinoline-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester), 2-{[2-bis(carboxymethyl)amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline tetrakis (acetoxymethyl) ester	A cell-permeant acetoxymethyl ester of the high-affinity fluorescent calcium indicator quin-2. Used for fluorescent determination of free calcium in intact cells. Penetrates cell membranes and undergoes enzymatic hydrolysis to quin-2 in the cytoplasm. Analysis can be made by fluorescence microscopy and flow cytometry (Mahanes *et al.*, 1986; Zhang & Wu, 1996)
	Fluo-3/AM; 4-(6-acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthenyl)-4′-methyl-2,2′(ethylenedioxy)dianiline-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl) ester	Cell permeable fluorescent indicator of intracellular Ca^{2+}; non-fluorescent until it is hydrolyzed intracellularly and/or in the presence of Ca^{2+}. Analysis can be made by confocal and fluorescence microscopy and flow cytometry (Merritt *et al.*, 1990; Del Olmo *et al.*, 2013)
	Indo-1, AM; 4-(6-carboxy-2-indolyl)-4′-methyl-2,2′-(ethylenedioxy) dianiline-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl) ester	A cell-permeant, UV light-excitable, ratiometric Ca^{2+} indicator. Analysis can be made by fluorescence microscopy, microphotometry and flow cytometry (Collin *et al.*, 2000; Bailey & Macardle, 2006)

Membrane potential and mitochondrial activity are indicators of mitochondrial function and are related, however it is important to note that these parameters cannot be confused, as the mitochondria can maintain their redox processes by electron transport even with low membrane potential (Chen, 1988; Terada, 1990). Therefore, evaluation of these two parameters can be used in a complementary form.

Furthermore, it is possible to measure mitochondria calcium levels, as this mineral is considered to be the central regulator of oxidative phosphorylation (Irvine and Aitken, 1986; McCormack & Denton, 1993). Calcium measurement in spermatozoa has been reported by the use of the fluorescent probes Quin-2 AM (Irvine & Aitken, 1986), fluo-3/AM (Giojalas, 1998; Harrison *et al.*, 1993) and indo-1AM (Brewis *et al.*, 2000). However, it would ideal to measure intramitochondrial calcium, as well as create reference indices, considering that calcium has other functions in the cell such as its role in sperm capacitation (Breitbart, 2002).

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

Although these assessments are indicative of mitochondria function, these techniques cannot be applied to quantify energy efficiency of sperm cells. Studies aimed at the evaluation of sperm energy metabolism using measurement of ATP levels are important to complement the mitochondria status assessment (Mukai and Okuno, 2004). High-performance liquid chromatography (Samizo *et al.*, 2001) or dosage by commercial kits are among the methods that can be used to measure the ATP and ADP levels (Perchec *et al.*, 1995). Measurement of ATP and ADP molecules was performed in several species such as mice (Mukai & Okuno, 2004), birds (Rowe *et al.*, 2013) and humans. However, more studies are necessary to develop indexes between production and ATP consumption, and relate these with sperm function.

Conclusion

In conclusion, there are still several questions covering the real contribution of the mitochondrial metabolism in sperm function in each species, although it is clear that this organelle can affect reproductive processes both positively and negatively (Amaral *et al.*, 2013). Moreover, as mitochondria are the main ROS source and sperm are extremely susceptible to oxidative damage (Nichi *et al.*, 2007b; Vernet *et al.*, 2004), the development of studies aimed at the prevention of mitochondrial dysfunction in sperm cells is extremely important, such as the regarding improvement of mechanisms to reduce ROS release or inactivation of mechanisms for a better mitochondrial function.

Acknowledgements. The authors thank the Department of Animal Reproduction of University of São Paulo for the knowledge designed to compile the data in this review.

Financial support. The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, project: 2017/13090-9), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support for the research mentioned in this review.

Conflicts of interest. None of the authors has any conflict of interest to declare.

References

Agarwal A, Nallella KP, Allamaneni SSR and Said TM (2004) Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod BioMed Online* **8**, 616–27.

Agarwal A, Virk G, Ong C and du Plessis SS (2014) Effect of oxidative stress on male reproduction. *World J Mens Health* **32**, 1–17.

Aitken RJ (2006) Sperm function tests and fertility. *Int J Androl* **29**, 69–75.

Aitken RJ, Paterson M, Fisher H, Buckingham DW and van Duin M (1995) Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci* **108**, 2017–25.

Aitken RJ, Ryan AL, Baker MA and McLaughlin EA (2004) Redox activity associated with the maturation and capacitation of mammalian spermatozoa. *Free Rad Biol Med* **36**, 994–1010.

Alvarez JG, Touchstone JC, Blasco L and Storey BT (1987) Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa superoxide dismutase as major enzyme protectant against oxygen toxicity. *J Androl* **8**, 338–48.

Amaral A, Lourenço B, Marques M and Ramalho-Santos J (2013) Mitochondria functionality and sperm quality. *Reproduction* **146**, R163–74.

Askari HA, Check JH, Peymer N and Bollendorf A (1994) Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. *Syst Biol Reprod Med* **33**, 11–5.

Bailey S and Macardle PJ (2006) A flow cytometric comparison of indo-1 to fluo-3 and Fura Red excited with low power lasers for detecting Ca^{2+} flux. *J Immunol Methods* **311**, 220–5.

Barros P (2007) Estresse oxidativo e integridade do DNA em sêmen resfriado de gato-do-mato-pequeno (*Leopardus tigrinus* SCHREBER 1775).

Bilodeau J.-F, Chatterjee S, Sirard M.-A and Gagnon C (2000) Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev* **55**, 282–8.

Bilodeau JF, Blanchette S, Gagnon C and Sirard MA (2001) Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* **56**, 275–86.

Blumer CG, Restelli AE, Giudice P.T.D, Soler TB, Fraietta R, Nichi M, Bertolla RP and Cedenho AP (2012) Effect of varicocele on sperm function and semen oxidative stress. *BJU Int* **109**, 259–65.

Brand MD and Esteves TC (2005) Physiological functions of the mitochondrial uncoupling proteins, UCP2 and, UCP3. *Cell Metab* **2**, 85–93.

Breitbart H (2002) Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol Cell Endocrinol* **187**, 139–44.

Brewis IA, Morton IE, Mohammad SN, Browes CE and Moore H.D.M (2000) Measurement of intracellular calcium concentration and plasma membrane potential in human spermatozoa using flow cytometry. *J Androl* **21**, 238–49.

Celeghini E.C.C, De Arruda RP, De Andrade A.F.C, Nascimento J and Raphael CF (2007) Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma., acrosomal and mitochondrial membranes. *Reprod Domest Anim* **42**, 479–88.

Chazotte B (2011) Labeling mitochondria with MitoTracker dyes. *Cold Spring Harb Protoc* **8**, 990–2.

Chen LB (1988) Mitochondrial membrane potential in living cells. *Ann Rev Cell Biol* **4**, 155–81.

Christen R, Schackmann RW and Shapiro BM (1983) Metabolism of sea urchin sperm., Interrelationships between intracellular pH, ATPase activity and mitochondrial respiration. *J Biol Chem* **258**, 5392–9.

Collin S, Sirard MA, Dufour M and Bailey JL (2000) Sperm calcium levels and chlortetracycline fluorescence patterns are related to the *in vivo* fertility of cryopreserved bovine semen. *J Androl.* **21**, 938–43.

Copeland WC (2002) *Mitochondrial DNA*, Springer.

Cummins J (1998) Mitochondrial, DNA in mammalian reproduction. *Rev Reprod* **3**, 172–82.

de Lamirande E and Cagnon C (1993) Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Rad Biol Med* **14**, 157–166.

de Lamirande E, Jiang H, Zini A, Kodama H and Gagnon C (1997) Reactive oxygen species and sperm physiology. *Rev Reprod* **2**, 48–54.

de Lamirande E.V.E, Tsai C, Harakat A and Gagnon C (1998) Involvement of reactive oxygen species in human sperm acrosome reaction induced by A23187, lysophosphatidylcholine, and biological fluid ultrafiltrates. *J Androl* **19**, 585–94.

Del Olmo E, Bisbal A, Maroto-Morales A, García-Alvarez O, Ramon M, Jimenez-Rabadan P, Martínez-Pastor F, Soler AJ, Garde JJ and Fernández-santos MR (2013) Fertility of cryopreserved ovine semen is determined by sperm velocity. *Anim Reprod Sci* **138**, 102–9.

Dorweiler B, Pruefer D, Andrasi T, Maksan S, Schmiedt W, Neufang A and Vahl C (2007) Ischemia-reperfusion injury. *Eur J Trauma Emerg Surg* **33**, 600–12.

Fernández-Santos MR, Martínez-Pastor F, García-Macías V, Esteso MC, Soler AJ, Paz P, Anel L and Garde JJ (2007) Sperm characteristics and, D. NA integrity of, Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. *J Androl* **28**, 294–305.

Ford W.C.L (2006) Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round? *Hum Reprod Update* **12**, 269–74.

Forster S, Thumser AE, Hood SR and Plant N (2012) Characterization of rhodamine-123 as a tracer dye for use in *in vitro* drug transport assays. *PLoS One* **7**, e33253.

Gallon F, Marchetti C, Jouy N and Marchetti P (2006) The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. *Fertil Steril* **86**, 1526–30.

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

García-Rivas G, de J, Carvajal K, Correa F and Zazueta C (2006) Ru360, a specific mitochondrial calcium uptake inhibitor., improves cardiac post-ischaemic functional recovery in rats *in vivo*. *Brit J Pharmacol* **149**, 829–37.

Garner DL, Thomas CA, Joerg HW, DeJarnette JM and Marshall CE (1997) Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biol Reprod* **57**, 1401–6.

Gillan L, Evans G and Maxwell WMC (2005) Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology* **63**, 445–57.

Giojalas LC (1998) Correlation between response to progesterone and other functional parameters in human spermatozoa. *Fertil Steril* **69**, 107–11.

Graham JK, Kunze E and Hammerstedt RH (1990) Analysis of sperm cell viability., acrosomal integrity, and mitochondrial function using flow cytometry. *Biol Reprod* **43**, 55–64.

Gunter TE, Yule DI, Gunter KK, Eliseev RA and Salter JD (2004) Calcium and mitochondria. *FEBS Lett* **567**, 96–102 2004.

Hallap T, Nagy S, Jaakma U, Johannisson A and Rodriguez-Martinez H (2005) Mitochondrial activity of frozen–thawed spermatozoa assessed by MitoTracker Deep Red 633. *Theriogenology* **63**, 2311–22.

Halliwell B (1999) *Free Radicals in Biology and Medicine*, Oxford University Press.

Halliwell B and Gutteridge J (1985) *Free Radicals in Biology and Medicine*, Pergamon.

Hammerstedt RH, Graham JK and Nolan JP (1990) cryopreservation of mammalian sperm: what we ask them to survive. *J Androl* **11**, 73–88.

Harrison R.A.P, Mairet B and Miller N.G.A (1993) Flow cytometric studies of bicarbonate-mediated, Ca^{2+} influx in boar sperm populations. *Mol Reprod Dev* **35**, 197–208.

Herzog V and Fahimi HD (1973) A new sensitive colorimetric assay for peroxidase using 3,3′-diaminobenzidine as hydrogen donor. *Anal Biochem* **55**, 554–62.

Holt WV (2000) Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology* **53**, 47–58.

Hrudka F (1987) Cytochemical and ultracytochemical demonstration of cytochrome c oxidase in spermatozoa and dynamics of its changes accompanying ageing or induced by stress. *Int J Androl* **10**, 809–28.

Hu CH, Zhuang XJ, Wei YM, Zhang M, Lu SS, Lu YG, Yang XG and Lu KH (2017) comparison of mitochondrial function in boar and bull spermatozoa throughout cryopreservation based on JC-1 staining. *Cryo Lett* **38**, 75–9.

Irvine DS and Aitken RJ (1986) Measurement of intracellular calcium in human spermatozoa. *Gamete Res* **15**, 57–71.

John J.C.S, Jokhi RP and Barratt C.L.R (2005) The effect of mitochondrial genetics on male infertility. *Int J Androl* **28**, 65–73.

Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S and Hoshi K (2002) Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. *Asian J Androl* **4**, 97–104.

Kasianowicz J, Benz R and McLaughlin S (1984) The kinetic mechanism by which CCCP (carbonyl cyanidem-chlorophenylhydrazone) transports protons across membranes. *J Membr Biol* **82**, 179–90.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA and Aitken RJ (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* **93**, 3199–207.

Krzyzosiak J, Molan P and Vishwanath R (1999) Measurements of bovine sperm velocities under true anaerobic and aerobic conditions. *Anim Reprod Sci* **55**, 163–73.

Lardy H, Winchester B and Phillips P (1945) The respiratory metabolism of ram spermatozoa. *Arch Biochem* **6**, 33–40.

Lin MT and Beal MF (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **443**, 787–95.

Losano JD, Padín JF, Méndez-López I, Angrimani DS, García AG and , Barnabe VH et al. 2017a) The stimulated glycolytic pathway is able to maintain ATP levels and kinetic patterns of bovine epididymal sperm subjected to mitochondrial uncoupling. oxidative medicine and cellular longevity. *Oxid Med Cell Longev* **2017**, 1682393.

Losano J, Angrimani D, Dalmazzo A, Rui B, Brito M and , Mendes C et al. 2017b) Effect of mitochondrial uncoupling and glycolysis inhibition on ram sperm functionality. *Reprod Domest Anim* **52**, 289–97.

Lowell BB and Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384–7.

Machado-Oliveira G, Lefièvre L, Ford C, Herrero MB, Barratt C, Connolly TJ, Nash K, Morales-García A, Kirkman-Brown J and Publicover S (2008) Mobilisation of Ca^{2+} stores and flagellar regulation in human sperm by *S*-nitrosylation: a role for NO synthesised in the female reproductive tract. *Development* **135**, 3677–86.

Mahanes MS, Ochs DL and Eng LA (1986) Cell calcium of ejaculated rabbit spermatozoa before and following *in vitro* capacitation. *Biochem Biophys Res Commun* **134**, 664–70.

Mailloux RJ and Harper M.-E (2011) Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Rad Biol Med* **51**, 1106–15.

Marchetti C, Jouy N, Leroy-Martin B, Defossez A, Formstecher P and Marchetti P (2004) Comparison of four fluorochromes for the detection of the inner mitochondrial membrane potential in human spermatozoa and their correlation with sperm motility. *Hum Reprod* **19**, 2267–76.

Marchetti C, Obert G, Deffosez A, Formstecher P and Marchetti P (2002) Study of mitochondrial membrane potential reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod* **17**, 1257–65.

Margulis L (1970) *Origin of Eukaryotic Cells. Evidence and Research Implications for a Theory of the Origin and Evolution of Microbial, Plant, and Animal Cells on the Precambrian Earth*. Yale, University, Press: New Haven.

McCormack JG and Denton RM (1993) Mitochondrial Ca^{2+} transport and the role of intramitochondrial Ca^{2+} in the regulation of energy metabolism. *Dev Neurosci* **15**, 165–73.

McCormack JG, Halestrap AP and Denton RM (1990) Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* **70**, 391–425.

Meijer J and Fentener, Van Vlissingen J (1993) *Gross structure and development of reproductive organs*. In Reproduction in Domesticated Animals, (ed. G.L. King), pp. 9–26. World Animal, Science-B9. Amsterdam: Elsevier Science Publishers.

Merritt JE, McCarthy SA, Davies MP and Moores KE (1990) Use of fluo-3 to measure cytosolic Ca^{2+} in platelets and neutrophils, loading cells with the dye, calibration of traces., measurements in the presence of plasma, and buffering of cytosolic Ca^{2+}. *Biochem J* **269**, 513–9.

Modica-Napolitano JS and Singh KK (2004) Mitochondrial dysfunction in cancer. *Mitochondrion* **4**, 755–62.

Mukai C and Okuno M (2004) glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biol Reprod* **71**, 540–7.

Nascimento JM, Shi LZ, Tam J, Chandsawangbhuwana C, Durrant B, Botvinick EL and Berns MW (2008) Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging., laser tweezers., and real-time automated tracking and trapping. *J Cell Physiol* **217**, 745–51.

Nelson DL and Cox MM (2008) *Principles of Biochemistry*, 5th edn, New York, NY: W.H. Freeman and Company.

Nevo AC and Rikmenspoel R (1970) Diffusion of ATP in sperm flagella. *J Theoret Biol* **26**, 11–8.

Nichi M, Bols P.E.J, Züge RM, Barnabe VH, Goovaerts I.G.F, Barnabe RC and Cortada C.N.M (2006) Seasonal variation in semen quality in *Bos indicus* and *Bos taurus* bulls raised under tropical conditions. *Theriogenology* **66**, 822–8.

Nichi M, Goovaerts IG, Cortada CN, Barnabe VH, De Clercq JB and Bols PE (2007a) Roles of lipid peroxidation and cytoplasmic droplets on *in vitro* fertilization capacity of sperm collected from bovine epididymides stored at 4 and 34 degrees C. *Theriogenology* **67**, 334–40.

Nichi M, Goovaerts IGF, Cortada C.N.M, Barnabe VH, De Clercq J.B.P and Bols P.E.J (2007b) Roles of lipid peroxidation and cytoplasmic droplets on *in vitro* fertilization capacity of sperm collected from bovine epididymides stored at 4 and 34°C. *Theriogenology* **67**, 334–40.

Nordberg J and Arnér E.S.J (2001) Reactive oxygen species., antioxidants., and the mammalian thioredoxin system. *Free Rad Biol Med* **31**, 1287–312.

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press

O'Connell M, McClure N and Lewis S.E.M (2002) The effects of cryopreservation on sperm morphology., motility and mitochondrial function. *Hum Reprod* **17**, 704–9.

Paul C, Teng S and Saunders P.T.K (2009) A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death. *Biol Reprod* **80**, 913–9.

Pariz JR and Hallak J (2016) Effects of caffeine supplementation in post-thaw human semen over different incubation periods. *Andrologia* **48**, 961–6.

Penefsky HS (1985) Mechanism of inhibition of mitochondrial adenosine triphosphatase by dicyclohexylcarbodiimide and oligomycin: relationship to ATP synthesis. *Proc Natl Acad Sci USA* **82**, 1589–93.

Perchec G, Jeulin C, Cosson J, Andre F and Billard R (1995) Relationship between sperm ATP content and motility of carp spermatozoa. *J Cell Sci* **108**, 747–53.

Piccoli C, Scrima R, D'Aprile A, Ripoli M, Lecce L, Boffoli D and Capitanio N (2006) Mitochondrial dysfunction in hepatitis C virus infection. *Biochim Biophys Acta* **1757**, 1429–37.

Poot M, Zhang YZ, Krämer JA, Wells KS, Jones LJ, Hanzel DK, Lugade AG, Singer VL and Haugland RP (1996) Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J Histochem Cytochem* **44**, 1363–72.

Ramalho-Santos J, Varum S, Amaral S, Mota PC, Sousa AP and Amaral A (2009) Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Hum Reprod Update* **15**, 553–72.

Rasola A and Geuna M (2001) A flow cytometry assay simultaneously detects independent apoptotic parameters. *Cytometry* **45**, 151–7.

Reyes JG, Farias JG, Henríquez-Olavarrieta S, Madrid E, Parraga M, Zepeda AB and Moreno RD (2012) The hypoxic testicle: physiology and pathophysiology. *Oxid Med Cell Longev* **2012**, 929285.

Rowe M, Laskemoen T, Johnsen A and Lifjeld JT (2013) Evolution of sperm structure and energetics in passerine birds. *Proc Biol Sci* **280** (1753):20122616.

Ruiz-Pesini E, Lapeña A.-C, Díez-Sánchez C, Pérez-Martos A, Montoya J, Alvarez E, Díaz M, Urriés A, Montoro L, López-Pérez MJ and Enríquez JA (2000) Human mtDNA haplogroups associated with high or reduced spermatozoa motility. *Am J Hum Genet* **67**, 682–96.

Samizo K, Ishikawa R, Nakamura A and Kohama K (2001) A highly sensitive method for measurement of myosin atpase activity by reversed-phase high-performance liquid chromatography. *Anal Biochem* **293**, 212–5.

Saraste M (1999) Oxidative phosphorylation at the fin de siècle. *Science* **283**, 1488–93.

Sariozkan S, Bucak MN, Tuncer PB, Ulutas PA and Bilgen A (2009) The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. *Cryobiology* **58**, 134–8.

Schober D, Aurich C, Nohl H and Gille L (2007) Influence of cryopreservation on mitochondrial functions in equine spermatozoa. *Theriogenology* **68**, 745–54.

Sherer TB, Betarbet R, Testa CM, Seo BB, Richardson JR, Kim JH, Miller GW, Yagi T, Matsuno-Yagi A and Greenamyre JT (2003) Mechanism of toxicity in rotenone models of Parkinson's disease. *J Neurosci* **23**, 10756–64.

Simões R, Feitosa WB, Siqueira A.F.P, Nichi M, Paula-Lopes FF, Marques MG, Peres MA, Barnabe VH, Visintin JA and Assumpção M.E.O (2013) Influence of bovine sperm, DNA fragmentation and oxidative stress on early embryo *in vitro* development outcome. *Reproduction* **146**, 433–41.

Slater EC (1973) The mechanism of action of the respiratory inhibitor, antimycin. *Biochim Biophys Acta* **301**, 129–54.

Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD and Chen LB (1991) Intracellular heterogeneity in mitochondrial membrane potentials revealed by a, J.-aggregate-forming lipophilic cation, J.C-1. *Proc Natl Acad Sci USA* **88**, 3671–5.

St John J, Bowles EJ and Amaral A (2006) Sperm mitochondria and fertilisation. *Soc Reprod Fertil Suppl***65**, 399–416.

St John JC (2002) The transmission of mitochondrial, DNA following assisted reproductive techniques. *Theriogenology* **57**, 109–23.

Storey BT (2008) Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int J Dev Biol* **52**, 427.

Szalai G, Krishnamurthy R and Hajnóczky G (1999) Apoptosis driven by IP_3-linked mitochondrial calcium signals. *EMBO J* **18**, 6349–61.

Taylor K, Roberts P, Sanders K and Burton P (2009) Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. *Reprod BioMed Online* **18**, 184–9.

Terada H (1990) Uncouplers of oxidative phosphorylation. *Environ Health Perspect* **87**, 213.

Territo PR, French SA, Dunleavy MC, Evans FJ and Balaban RS (2001) Calcium activation of heart mitochondrial oxidative phosphorylation: rapid kinetics of mv, O_2, NADH, and light scattering. *J Biol Chem* **276**, 2586–99.

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA and Clark AM (2009) Cryopreservation-induced human sperm, DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* **24**, 2061–70.

Thu LT, Ahn JR and Woo S.-H (2006) Inhibition of L-type Ca^{2+} channel by mitochondrial, Na^+–Ca^{2+} exchange inhibitor CGP-37157 in rat atrial myocytes. *Eur J Pharmacol* **552**, 15–9.

Travis AJ, Foster JA, Rosenbaum NA, Visconti PE, Gerton GL, Kopf GS and Moss SB (1998) Targeting of a germ cell-specific type 1 hexokinase lacking a porin-binding domain to the mitochondria as well as to the head and fibrous sheath of murine spermatozoa. *Mol Biol Cell* **9**, 263–76.

Troiano L, Granata ARM, Cossarizza A, Kalashnikova G, Bianchi R, Pini G, Tropea F, Carani C and Franceschi C (1998) mitochondrial membrane potential and DNA stainability in human sperm cells: a flow cytometry analysis with implications for male infertility. *Exp Cell Res* **241**, 384–93.

Turner RM (2003) Tales from the tail: what do we really know about sperm motility? *J Androl* **24**, 790–803.

Vernet P, Aitken RJ and Drevet JR (2004) Antioxidant strategies in the epididymis. *Mol Cell Endocrinol* **216**, 31–9.

Vincent AM, Olzmann JA, Brownlee M, Sivitz WI and Russell JW (2004) Uncoupling proteins prevent glucose-induced neuronal oxidative stress and programmed cell death. *Diabetes* **53**, 726–34.

Wang G, Guo Y, Zhou T, Shi X, Yu J, Yang Y, Wu Y, Wang J, Liu M, Chen X, Tu W, Zeng Y, Jiang M, Li S, Zhang P, Zhou Q, Zheng B, Yu C, Zhou Z, Guo X and Sha J (2013) In-depth proteomic analysis of the human sperm reveals complex protein compositions. *J Proteomics* **79**, 114–22.

Wang X, Sharma RK, Gupta A, George V, Thomas Jr AJ, Falcone T and Agarwal A (2003) Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study. *Fertil Steril* **80 (Suppl 2)**, :844–50.

White IG and Wales RG (1961) Comparison of epididymal and ejaculated semen of the ram. *J Reprod Fertil* **2**, 225–37.

Wojcik C, Sawicki W, Marianowski P, Benchaib M, Czyba JC and Guerin JF (2000) Cyclodextrin enhances spermicidal effects of magainin-2-amide. *Contraception* **62**, 99–103.

Zhang CL and Wu BJ (1996) Development of calcium fluorescent probes and their application in life sciences. *Progr Physiol* **27**, 37–42.

Zapzalka DM, Redmon JB and Pryor JL (1999) A survey of oncologists regarding sperm cryopreservation and assisted reproductive techniques for male cancer patients. *Cancer* **86**, 1812–7.

https://doi.org/10.1017/S0967199418000242 Published online by Cambridge University Press