JOÃO DIEGO DE AGOSTINI LOSANO

Papel da mitocôndria na homeostase oxidativa e na funcionalidade de espermatozoides ovinos submetidos à criopreservação

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Orientador:
Prof. Dr. Marcilio Nichi

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Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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Prof. Dr.____

Instituição:___________________________ Julgamento:________________________

Prof. Dr.____

Instituição:___________________________ Julgamento:________________________

Prof. Dr.____

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DEDICATÓRIA

Dedico esta dissertação,

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Epígrafe

“You, me, or nobody is gonna hit as hard as life. But it ain't about how hard you hit. It's about how hard you can get hit and keep moving forward. How much you can take and keep moving forward. That's how winning is done!”

Sylvester Stallone**, **Rocky Balboa

RESUMO

LOSANO, J. D. A. **Papel da mitocôndria na homeostase oxidativa e na funcionalidade de espermatozoides ovinos submetidos à criopreservação**. [Role of mitochondria in oxidative homeostasis and functionality of ram sperm submitted to cryopreservation]. 2016. 111 f. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

Estudos têm demonstrado a importância da mitocôndria para a funcionalidade do espermatozoide, referindo-a como a principal fonte de energia para a motilidade e a homeostase celular. No entanto, para algumas espécies animais, estudos recentes indicam que a glicólise parece ser o principal mecanismo de produção de ATP para a motilidade espermática, superior à fosforilação oxidativa. Em ovinos estudos envolvendo o metabolismo energético do espermatozoide são necessários não apenas pelo seu interesse zootécnico, mas também como modelo experimental para bovino, espécie na qual este mecanismo é também pouco conhecido. Apesar da importância da mitocôndria para o metabolismo celular durante a fosforilação oxidativa, são produzidos metabólitos denominados Espécies Reativas de Oxigênio, as quais possuem um papel fundamental em diversos processos fisiológicos. No entanto, um eventual desequilíbrio entre a produção de EROs e os mecanismos antioxidantes caracteriza o estresse oxidativo, que pode ser letal para as células espermáticas. Ademais, estudos anteriores relacionam as disfunções mitocondriais causadas pela criopreservação espermática ao estresse oxidativo e a diminuição da atividade mitocondrial. Desta forma, acreditamos que injúrias mitocondriais durante a criopreservação são a origem da produção excessiva de fatores pró-oxidativos e, em última análise, causadores dos danos espermáticos pós-descongelação e diminuição da motilidade. Em face do exposto, a hipótese central do presente experimento é que o espermatozoide ovino, após despolarização mitocondrial por desacoplamento da fosforilazação oxidativa e suplementação para a glicólise, é capaz de manter a produção de ATP e, consequentemente, a motilidade espermática. Ainda, um leve desacoplamento mitocondrial é benéfico para os espermatozoides durante a criopreservação por diminuir as crioinjúrias mediadas por disrupções mitocondriais. Em relação aos nossos estudos de fisiologia, observamos no experimento 1 que os espermatozoides ovinos, mesmo apresentando suas mitocôndrias despolarizadas são capazes de manter a motilidade total. Este

resultado nos sugere que a via glicolítica possivelmente é capaz de manter a motilidade espermática. Por outro lado, o desacolpamento mitocondrial alterou os padrões do movimento espermático, nos sugerindo que a mitocôndria possui um papel mais importante na qualidade do movimento espermático do que na motilidade total. Ainda, no experimento 2 observamos que a via glicolítica, após ser estimulada, é capaz de manter os níveis de ATP, os padrões de cinética espermática e a homeostase oxidativa dos espermatozoides epididimários bovinos submetidos ao desacoplamento mitocondrial. Em relação ao nosso estudo aplicado (experimento 3), observamos que os espermatozoides ovinos criopreservados submetidos à um leve desacoplamento mitocondrial concomitantemente à estimulação da via glicolítica apresentaram maior motilidade, menor peroxidação lipídica, menor susceptibilidade da cromatina à denaturação ácida e maior potencial de membrana mitocondrial. Estes resultados nos indicam que um leve desacoplamento mitocondrial durante a criopreservação espermática é capaz de proteger as mitocôndrias contra as crioinjúrias e consequentemente melhorar a qualidade espermática pós-descongelação.

Palavras-chave: Espermatozoides. Ruminantes. Metabolismo espermático. Glicólise. Fosforilação oxidativa

ABSTRACT

LOSANO, J. D. A. **Role of mitochondria in oxidative homeostasis and functionality of ram sperm submitted to cryopreservation**. [Papel da mitocôndria na homeostase oxidativa e na funcionalidade de espermatozoides ovinos submetidos à criopreservação]. 2016. 111 f. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

Studies have demonstrated the importance of mitochondria in the sperm functionality, referring to it as the main source of energy for motility and cellular homeostasis. However, for some animal species, recent studies indicate that glycolysis seems to be the main mechanism ATP production for sperm motility, higher than the oxidative phosphorylation. In ovine studies involving energy metabolism of sperm are required not only for their livestock interest, but also as an experimental model for bovine species in which this mechanism is also unknown. Despite the importance of mitochondria for cellular metabolism during oxidative phosphorylation, they are produced metabolites called reactive oxygen species, which have a key role in many physiological processes. However, any imbalance between ROS and antioxidant mechanisms characterizes oxidative stress, which may be lethal for the sperm cells. Moreover, previous studies relate to mitochondrial dysfunction caused by oxidative stress on sperm cryopreservation and decreased mitochondrial activity. Thus, we believe that mitochondrial injury during cryopreservation are the source of excessive production of pro-oxidative factors and ultimately, causing the post-thaw sperm damage and decrease in motility. In view of the above, the central hypothesis of this experiment is that the ovine sperm after mitochondrial depolarization by uncoupling of oxidative phosphorylation and glycolysis supplementation is capable of maintaining the ATP production and consequently sperm motility. Additionally, a mild mitochondrial uncoupling is beneficial for spermatozoa during cryopreservation by decreasing the cryoinjuries mediated by mitochondrial disruption. Regarding our physiology studies, we observed in experiment 1 that the ovine sperm, even with their depolarized mitochondria are able to maintain total motility. This result suggests that the glycolytic pathway is possibly able to maintain motility. Moreover, the fact that mitochondrial uncoupling altered sperm movement patterns suggests that mitochondria has a more important role in the quality of sperm kinetic than the total motility. Furthermore, in the experiment 2 we observed that glycolytic pathway, after being stimulated, is able to

maintain ATP levels, sperm kinetics patterns and oxidative homeostasis of bovine epididymal spermatozoa submitted to mitochondrial uncoupling. Regarding our applied study (Experiment 3), we observed that cryopreserved ovine sperm submitted to mild mitochondrial uncoupling concurrently with glycolysis stimulation showed increased motility, lower lipid peroxidation, lower susceptibility of chromatin to acid denaturation and higher mitochondrial membrane potential. These results indicate that a slight mitochondrial uncoupling during sperm cryopreservation can protect mitochondria against cryoinjuries and hence improve the post-thaw spermatozoa quality.

Keywords: Spermatozoa. Ruminants. Sperm metabolism. Glycolysis. Oxidative Phosphorylation

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Introduction

1 INTRODUCTION

The nuclear power plant Chernobyl, located in the Ukraine and considered a worldwide reference on energy production, was capable of generating an amount of four megawatts of electric energy. In 1986, a serious accident in reactor no. 4 led to release of radioactive material equivalent to 400 times than was observed in the atomic bombing of Hiroshima. As a result, approximately 3.900.000 Km^2 of the European and Asian continents were contaminated with cesium - 137 (FAIRLIE; SUMNER, 2006). Despite the obvious difficulties on estimating the casualties directly or indirectly linked to the accident (FAIRLIE; SUMNER, 2006), millions of people were exposed to radioactive material leading to high incidence of mutation, several types of cancer, especially in the thyroid (KAZAKOV; DEMIDCHIK; ASTAKHOVA, 1992; KLUGBAUER et al., 1995), as well as infant leukemia after intrauterine exposure (PETRIDOU et al., 1996). Until now, some areas near the power plant cannot be inhabited due to isotopes still present in the environment.

Similarly to a nuclear power plant, mitochondria exhibit high energy production capacity; however, in situations which the structure of this organelle is compromised, the potential to release extremely toxic products is also injurious. Such toxic substances may lead to damages in 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 relation to the spermatozoa, several studies have referred mitochondria as the main source of energy, also playing important role on the cellular homeostasis maintenance and motility (TRAVIS et al., 1998; ST. JOHN, 2002). However, for some species, evidences suggest that glycolysis may be the main source of ATP production for sperm motility, superior to oxidative phosphorylation (MUKAI; OKUNO, 2004; FORD, 2006; NASCIMENTO et al., 2008).

Despite the importance of mitochondria to sperm metabolism, during oxidative phosphorylation are produced metabolites called reactive oxygen species (ROS), substances with important role on 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., 2007b).

As the main source of pro-oxidative factors, mitochondria has been found as crucial on the disruption of oxidative homeostasis (AGARWAL et al., 2014). In fact, several studies have demonstrated correlations between impaired mitochondrial activity with both oxidative stress and sperm DNA fragmentation, indicating a close relationship between these variables on the sperm damage pathogenesis (BARROS, 2007; NICHI et al., 2007a; BLUMER et al., 2012).

Since the Chernobyl accident, the main concern of nuclear energy specialists and the community in general is on the approaches to avoid the destruction caused by an eventual nuclear disaster. If it was possible, the deactivation of the power plant would probably avoid most of the damages prior a predictable stressful event. Similarly, the reversible inhibition of mitochondrial activity in situations where this organelle dysfunction is known (i.e., sperm cryopreservation) (O'CONNELL; MCCLURE; LEWIS, 2002; SARIOZKAN et al., 2009; THOMSON et al., 2009) would probably improve sperm viability by decreasing the amount of pro-oxidative factors available for release. Actually, a few studies have suggested that, for some cellular types, uncouplers of the oxidative phosphorylation are capable of reducing oxidative stress (VINCENT et al., 2004; MAILLOUX; HARPER, 2011).

This review aims to provide a brief introduction to cellular respiration, compile literature data about the role of mitochondria in oxidative homeostasis and sperm functionality as well as suggest some tools to assess sperm mitochondrial function.

Literature review

2 LITERATURE REVIEW - Sperm mitochondria: role in metabolism, oxidative homeostasis and functionality

2.1 THE MITOCHONDRIAL PARADOX: PHYSIOLOGICAL AND PATHOLOGICAL ROLE ON SPERMATOZOA

According to the endosymbiotic theory, millions of years ago, mitochondria was a prokaryotic unicellular organism. Formerly a free-living bacterium, mitochondria was capable to metabolizing oxygen in environment rich in carbon dioxide. After penetrate a host eukaryotic cell, incapable of metabolize oxygen, a symbiotic relationship was stablished, later originating a more complex organism capable of producing energy more efficiently than the previously available glycolysis (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).

2.2 THE ROLE OF MITOCHONDRIA ON ATP PRODUCTION AND SPERM PHSYSIOLOGY

Studies have demonstrated the main role of mitochondria on sperm functionality, referring this organelle as the main source of ATP for cellular homeostasis and motility (TRAVIS et al., 1998; ST. JOHN, 2002). However, such role on sperm metabolism has been a matter of debate. Mukai and Okuno (2004), when inhibiting sperm mitochondrial activity in mice, concomitantly to the supplementation of the glycolytic pathway, observed motility, ATP production and flagellar beat remained unaltered. However, when glycolysis was inhibited and oxidative phosphorylation was stimulated, observed the flagellar beat and ATP production were drastically reduced, suggesting the glycolysis is more relevant than oxidative

phosphorylation on murine sperm energetic metabolism. Similar results were observed by Nascimento et al. (2008) in human sperm. The authors suggest that, despite the important contribution of oxidative phosphorylation for ATP production, glycolysis is the primary source of energy in human sperm. On the other hand, studies have observed the opposite effect, when the sperm samples were incubated with inhibitors of the enzymatic electron transport complexes, it decreased human sperm motility. However, it was not verified the glycolysable substrates influence in these studies (RUIZ-PESINI et al., 2000; JOHN; JOKHI; BARRATT, 2005).

It is well known that mitochondria have a main importance on sperm functionality as several researches showed a relationship between mitochondrial functional role and fertilizing capacity (MARCHETTI et al., 2002; MARCHETTI et al., 2004; GALLON et al., 2006; ST JOHN; BOWLES; AMARAL, 2006). Nonetheless, it is not clear how mitochondria contribute to the sperm energetic capacity. The variability of research results suggests that such cell organelle may have distinct contributions to sperm metabolism depending on experimental biological 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 constantly described (MUKAI; OKUNO, 2004). Lardy, Winchester and Phillips (1945) first showed that mitochondrial inhibition leads to asthenospermia. However, glucose supplementation to sperm sample the sperm motility was reacquired. In addition, White e Wales (1961) observed that ovine sperm maintain motility through two parallel mechanisms of energetic generation, i.e., glycolysis and oxidative phosphorylation. Moreover, Krzyzosiak, Molan and Vishwanath (1999) also observed bovine sperm are capable of maintaining similar motility patterns on both aerobic and anaerobic conditions assuming that glycolysable substrates are available. Furthermore, previous studies suggest 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 on flagellar beat in this region (NEVO; RIKMENSPOEL, 1970; TURNER, 2003).

2.2.1 Role of calcium on mitochondrial function

A hypothesis to the main regulatory mechanisms of oxidative phosphorylation considers ADP and inorganic phosphate as feedbackers for ATP synthesis, through several cellular kinases. Therefore, an interesting analogy can be employed with the economic model of supply and demand, being the ATP as the unit price for cellular energy. Such theory is supported by the fact that isolated mitochondria in suspension increased their ATP production when ADP and inorganic phosphate is supplemented in the presence of oxygen. Although the known “economic model of equilibrium”, recent studies have shown that ATP synthesis rate is not strictly controlled by such mechanism (GUNTER et al. 2004).

Mitochondrial calcium ([Ca^{2+}]m) has been referred as the central regulator of oxidative phosphorylation, acting as primary metabolic mediator for NADH production and the enzymatic complexes pyruvate dehydrogenase, isocitrate dehydrogenase and α- ketoglutarate dehydrogenase activity controler (MCCORMACK, JAMES; HALESTRAP; DENTON, 1990; MCCORMACK; DENTON, 1993). The [Ca^{2+}]m is also directly involved on ATP production, playing important role on ADP phosphorylation through the enzyme ATP-synthase (TERRITO et al., 2001). Moreover, mitochondrial calcium also participates on apoptotic mechanism of somatic cells, triggering the release of pro-apoptotic agents by the mitochondria (SZALAI; KRISHNAMURTHY; HAJNÓCZKY, 1999).

If on one hand, the participation of [Ca^{2+}]m on physiological processes of somatic cells is well stablished, on the other hand, the precise role of this ion on sperm mitochondria is still a matter of debate (AMARAL et al., 2013). From a proteomic approach, studies have identified sperm Mitochondrial Calcium Uniporters (MCU), proteins responsible for controlling mitochondrial calcium signalization, 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, mitochondrial activity of bulls´ hyperactivated sperm appears to be unregulated by calcium release. In this context, further studies are vital to stablish the real function calcium concentrations on mitochondrial physiology, reference values for [Ca^{2+}]m, and to correlate such

values with sperm function (IRVINE; AITKEN, 1986; RAMALHO-SANTOS et al., 2009; AMARAL et al., 2013).

2.2.2 Reactive oxygen species and the spermatozoa

During the aerobic cell metabolism, metabolites known as reactive oxygen species (ROS) are formed. Mitochondrial environment is rich in oxygen and electrons, and almost all of these electrons participating in the reduction of oxygen directly to water, the final product of oxidative phosphorylation. However, physiologically, some of these electrons escape from enzymatic complex of oxidative phosphorylation and bind to molecular oxygen, leading to the superoxide anion, first ROS generated. From this primary product, a reaction redox cascade occurs raising to other reactive oxygen species such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) respectively. Some of these ROS can be named free radicals because they have unpaired electrons in its last electron layer (FERREIRA; MATSUBARA, 1997; NORDBERG; ARNÉR, 2001).

The ROS produced by spermatozoa have a key role in many physiological processes, such as sperm hyperactivation (DE LAMIRANDE, EVE; CAGNON, 1993), sperm capacitation (AITKEN JOHN et al., 2004), acrosome reaction (DE LAMIRANDE et al., 1998), and interaction between spermatozoa and the zona pellucida (AITKEN et al., 1995), usually acting as physiological triggers. While ROS are formed by other mechanism such as glycolysis, mitochondria is the main source of ROS, with approximately 2% of consumed oxygen is converted to superoxide anion (KOPPERS et al., 2008).

A number of enzymatic and non-enzymatic antioxidants act synergistically to prevent excessive formation of these ROS, where each of these metabolites is inactivated by specific antioxidants. The enzyme superoxide dismutase (SOD) is considered the primary line of antioxidant defense acting through dismutation of two molecules of superoxide anion (O_2^-) forming an oxygen molecule and a hydrogen peroxide molecule (H_2O_2) (ALVAREZ et al., 1987). H_2O_2 can be destroyed by two antioxidants independent systems, the enzyme catalase and glutathione peroxidase / glutathione reductase system (NORDBERG; ARNÉR, 2001). If these two systems

fail, the H_2O_2 will react with an Fe^{2+} or Cu^+ molecule (a process known as Fenton reaction) and will form the hydroxyl radical (OH^-). This reactive oxygen species is considered the most reactive in biological systems, and can be destroyed by non-enzymatic antioxidants such as ascorbic acid and α-tocopherol (HALLIWELL; BARRY; GUTTERIDGE, 1985).

2.2.3 Mitochondrial disfunctions x spermatozoa

Despite the ROS physiological function, any imbalance in ROS production and antioxidant mechanisms characterized oxidative stress, which may be lethal for sperm cells (DE LAMIRANDE et al., 1997; AGARWAL et al., 2004). The sperm is particularly susceptible to oxidative stress, by owning an extremely small cytoplasm and consequently low antioxidant activity, and by also has high amount of polyunsaturated fatty acids (easily oxidized) in its membrane. Thus, this stress may cause damage to different sperm structures, such as plasma and acrosomal membranes, mitochondria and sperm DNA. The spermatozoa is not able to restore these oxidative damage due to deficiency of cytoplasmic repair enzymes (VERNET; AITKEN; DREVET, 2004; NICHI et al., 2007a; AGARWAL et al., 2014).

Once the mitochondria is the major source of pro-oxidative agents, it is suggested therefore that this organelle dysfunctions have a fundamental role in the oxidative imbalance affecting sperm function (AGARWAL et al., 2014). Wang et al. (2003) identified in sperm of infertile patients low mitochondrial membrane potential and high ROS production, probably as a consequence of such mitochondrial injury, suggesting that mitochondrial function can be a marker of male fertility. In fact, other researchers observed changes in mitochondrial function in sperm derived from infertile men (TROIANO et al., 1998; GALLON et al., 2006). However, it has been identified sperm samples with high mitochondrial membrane potential in fertile patients (KASAI et al., 2002; MARCHETTI et al., 2002).

Studies performed in different species showed a negative correlation between both oxidative stress and high mitochondrial activity, as well as between the occurrence of this stress and the sperm DNA integrity, indicating that these variables are linked, leading a single pathogenic mechanism (BARROS, 2007; NICHI et al.,

2007a; BLUMER et al., 2012). In addition, correlations were also found between variables of the spermatic oxidative stress and lower blastocyst rates as well as increased rates of blastomeres with DNA damage, confirming a negative impact of seminal oxidative stress in the embryonic development *in vitro* (SIMÕES et al., 2013).

Mitochondrial disorders have multifactorial origins, and some mechanisms are not yet fully elucidated (AMARAL et al., 2013). Such changes can be caused even in the testis during spermatogenesis. It is known that testicular thermoregulatory mechanism is inefficient. It is believed that only 50% of the blood supply that reaches through the testicular artery supplying the testes, causing the male gonads working the edge of hypoxia (MEIJER; FENTENER VAN VLISSINGEN, 1993). The increase in the testis metabolism after any pathology that raises testicular temperature is not compensated by an increase in blood flow, causing testis hypoxic condition (PAUL; TENG; SAUNDERS, 2009). After the softening of this condition and the beginning of oxygenation, there is an increased production of reactive oxygen species generating the oxidative stress. This mechanism is known as ischemia-reperfusion injury (NICHI et al., 2006; REYES et al., 2012). The increased of ROS production in this mechanism is related to mitochondrial dysfunction and subsequent activation of enzymes that work as generators ROS systems, such as xanthine oxidase (XO). These mitochondrial changes are related to the lack of O_2 during ischemia, which leads to a depletion of ATP and a consequent mitochondrial injury. Moreover, the increased testicular temperature promotes an influx of calcium that is also related to changes in this organelle (DORWEILER et al., 2007; REYES et al., 2012).

Sperm cryopreservation is considered a key process in assisted reproduction techniques (HAMMERSTEDT; GRAHAM; NOLAN, 1990; ZAPZALKA; REDMON; PRYOR, 1999; HOLT, 2000). However it is known that this technique promotes a decrease in sperm quality, and some researchers observed that mitochondrial damage during cryopreservation is the source of excessive production of pro-oxidative factors and, ultimately, causing the post-thaw sperm damage and motility decreased (O'CONNELL; MCCLURE; LEWIS, 2002; SARIOZKAN et al., 2009; THOMSON et al., 2009). In addition, a decrease in antioxidant capacity after sperm cryopreservation was detected, further factor that predisposes these cells to oxidative stress (BILODEAU et al., 2000).

Thus, several studies have used antioxidant treatment in sperm samples submitted to cryopreservation, aiming the prevention of oxidative stress caused by

mitochondrial injuries (ASKARI et al., 1994; BILODEAU et al., 2001; FERNÁNDEZ-SANTOS et al., 2007; TAYLOR et al., 2009). However, it is suggested that a specific mitochondrial shield during cryopreservation for improving post-thaw sperm quality (SCHOBER et al., 2007). A possible alternative would be reduce mitochondrial activity, induced by uncouplers or inhibitors of oxidative phosphorylation during the cryopreservation process, for any mitochondrial dysfunction during this processrelease a lower pro-oxidative agents. In fact, the activities of some uncouplers were identified in physiological processes of somatic cells, and even acting in the oxidative stress reduction (VINCENT et al., 2004; BRAND; ESTEVES, 2005).

2.3 INHIBITORS AND UNCOUPLES OF OXIDATIVE PHOSPHORYLATION: ACTION MECHANISMS AND THEIR POSSIBLE APPLICATIONS

Inhibitors and uncouplers of oxidative phosphorylation have an essential role in the study of mitochondrial physiology, being widely used as a pharmacological tool. This was possible because there are many chemical compounds that inhibit the specific processes of oxidative phosphorylation. So inhibiting a single process is possible observe their role as well as the act of other mechanisms that are not inhibited (NELSON; COX, 2008).

Therefore it can inhibit some complex electron carriers as well as some mitochondrial channels. The rotenone (insecticide class), e.g., blocks the transfer of electrons from the complex I to ubiquinone, inhibiting, therefore, the overall process of oxidative phosphorylation (SHERER et al., 2003). Antimycin A, antibiotic produced by Streptomyces fungus, blocks the transport of electrons of the complex III to complex IV (SLATER, 1973). The cyanide finally inhibits electrons transport complex IV to oxygen. Furthermore, it is possible inhibit directly ATP synthesis, with oligomycin widely used in this process. This compound acts on the enzyme ATP - synthase, blocking the flow of protons through the F0 subunit of this enzyme to the mitochondrial matrix and consequently prevents the ATP synthesis (PENEFSKY, 1985). Besides the enzymatic complex inhibitors, there is also calcium channel

blockers, such as RU360, as well as Na+ / Ca2+pump inhibitors, such as CGP 37157 (DE J GARCÍA-RIVAS et al., 2006; THU; AHN; WOO, 2006).

Beyond these inhibitors, uncouplers of oxidative phosphorylation has been widely used not only as a tool to study cell physiology, but also as a possible therapeutic application (KASIANOWICZ; BENZ; MCLAUGHLIN, 1984). ATP synthesis occurs through coupling of two reactions, the electron transport and phosphorylation, as a result of proton gradient. This class of inhibitors uncouples these two reactions preventing or decreasing the ATP synthesis, however, the electron flow activity across the mitochondrial complexes are not inhibited, or even can be increased (TERADA, 1990). Most of these molecules are hydrophobic and have protonophore activity, i.e., depolarized mitochondrial membrane allowing the protons to return to the mitochondrial matrix and dissipate the mitochondrial membrane potential and pH difference, so inhibiting the driving proton force, essential for ATP synthesis (CHEN, 1988; TERADA, 1990).

Uncoupling proteins have been identified in some cell and related to some physiological roles such as in adaptive thermogenesis in brown adipose tissue. In addition, these proteins have been related in researches related to obesity, diabetes, neurodegenerative disease and aging (BRAND; ESTEVES, 2005). These studies occur due to some researches that found that mitochondrial uncouplers can control the ROS production by mitochondria and thus prevent oxidative stress, which is related to these diseases. Therefore, it is suggested the use of these proteins in cell therapy, to the treatment of these pathologies. (BRAND; ESTEVES, 2005; LOWELL; SHULMAN, 2005; LIN; BEAL, 2006; MAILLOUX; HARPER, 2011). The decreased of ROS production promoted by uncouplers is due to an increase in respiratory rate followed by a decrease in mitochondrial intermediates reduced states capable of donating single electrons to oxygen, thereby preventing the generation of primary ROS superoxide anion.

Despite the mitochondrial uncouplers be applied in the energy study of spermatozoa (MUKAI; OKUNO, 2004), still there is no evidence that these compounds can control the production of ROS by sperm mitochondria. However, the use of these substances can be interesting for the prevention of oxidative stress in seminal samples front of possible mitochondrial dysfunction. This treatment becomes attractive, especially for use in spermatozoa due to its high susceptibility to oxidative stress.

2.4 TOOLS FOR ASSESSING SPERM MITOCHONDRIAL FUNCTIONALITY

Due to the fact of sperm mitochondria be involved both in physiological as pathological processes, it is evident the importance of assessing the functionality of this organelle. Thus, the use of tools to evaluates the sperm mitochondrial function associated with other sperm analysis can approach 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; SCHACKMANN; SHAPIRO, 1983; HRUDKA, 1987; GRAHAM; KUNZE; HAMMERSTEDT, 1990).

The mitochondrial activity evaluation aims to infer the efficiency of electron transport between the enzymatic complexes, and thus redox processes involved in oxidative phosphorylation. Hrudka (1987), three decades ago it had already developed a cytochemical technique to evaluate this activity. This cytochemical assay is based on the oxidation of 3'3-diaminobenzidine (DAB) by Cytochrome-C, an enzyme involved in the electrons transport between the enzymatic complex. Later, some fluorescent probes arise with the same purpose, such as H2-CMXros and CMXros, commercially known as Mito Tracker Red® (POOT et al., 1996; WOJCIK et al., 2000; CELEGHINI et al., 2007).

Some fluorescent probes have also been developed to assess the mitochondrial membrane potential, such as JC-1 (iodide 5,5´,6,6´-tetracloro-1,1,3,3´tetraetilbenzimidazolilcarbocianine) (GARNER et al., 1997), Mito Tracker Green FM® (GILLAN; EVANS; MAXWELL, 2005) and Rodhamine 123® (GRAHAM; KUNZE; HAMMERSTEDT, 1990). The probes diffuse freely through the plasma membrane to the cytosol of the cell and accumulates electrophoretically in the mitochondrial matrix driven by the driving proton force, acting in accordance with the ability of mitochondria to pump protons from the matrix to the inter-membrane space (CHEN, 1988; GARNER et al., 1997; PICCOLI et al., 2006). Despite the membrane potential and activity mitochondrial are indicators of mitochondrial function and are related, cannot confounding between these parameters, since the mitochondria can maintain their redox processes by electron transport even with low membrane potential (CHEN, 1988; TERADA, 1990). Therefore, the evaluation of these two parameters can be used in a complementary form.

Furthermore, it is possible to measure the mitochondrial calcium levels, since this mineral is considered as the central regulator of oxidative phosphorylation (IRVINE; AITKEN, 1986; MCCORMACK; DENTON, 1993). The measurement of calcium in spermatozoa has been reported through the fluorescent probes Quin-2 AM (IRVINE; AITKEN, 1986), fluo-3/AM (HARRISON; MAIRET; MILLER, 1993; GIOJALAS, 1998) and indo-1AM (BREWIS et al., 2000). However, the ideal would be to measure the intramitochondrial calcium, as well as creating reference indices, since calcium has other functions in this cell, as sperm capacitation (BREITBART, 2002).

Although these assessments are indicative of mitochondrial function, through these techniques is not possible to quantify the energy efficiency of sperm cells. Therefore, studies aiming to study energy metabolism of the sperm measured ATP levels, complementing the assessment of mitochondrial status (MUKAI; OKUNO, 2004). Among the methods used to measure the levels of ATP and ADP, can be used in high performance liquid chromatography (SAMIZO et al., 2001) or dosage for commercial kits (PERCHEC et al., 1995). The measurement of ATP molecules and ADP have been performed in several species, such as mice (MUKAI; OKUNO, 2004) birds (ROWE et al., 2013) and human, however there is a need for more research to create indexes between production and consumption of ATP, and relate them with sperm function.

Therefore there is a need for further studies in several species to clarify the real contribution to the mitochondrial metabolism and sperm function, although it is clear that this organelle can impact both positively and negatively on the reproductive processes (AMARAL et al., 2013). The fact the mitochondria be the main ROS source, and the sperm be extremely susceptible to oxidative damage (VERNET; AITKEN; DREVET, 2004; NICHI et al., 2007a), it is extremely important researches aimed at the prevention of mitochondrial dysfunction in this cell, as well as the development of mechanisms to reduce the release of reactive oxygen species, or to inactivate these ROS if these disorders occur.

Chapter 1

3 CHAPTER 1: Effect of mitochondrial uncoupling and glycolysis inhibition on ram sperm functionality

ABSTRACT

Studies have demonstrated the importance of mitochondria to sperm functionality, as the main source of ATP for cellular homeostasis and motility. However, the role of mitochondria on sperm metabolism is still controversial. Studies indicate that, for some species, glycolysis may be the main mechanism for sperm energy production. For ram sperm, such pathway is not clear. Thus, we evaluated ram sperm in response to mitochondrial uncoupling and glycolysis inhibition aiming to assess the importance of each pathway for sperm functionality. Statistical analysis was performed by the SAS system for Windows, using the General Linear Model Procedure. Data was tested for residue normality and variance homogeneity. A $p<0.05$ was considered significant. Groups treated with the mitochondrial uncoupler CCCP showed a decrease in the percentage of cells with low mitochondrial activity and high mitochondrial membrane potential. We also observed that the highest CCCP concentration promotes a decrease on sperm susceptibility to lipid peroxidation. Regardless the lack of effect of CCCP on total motility, this substance induced significant alterations on sperm kinetics. Besides the interference of CCCP on spermatic movement patterns, it was also possible to observe such an effect in samples treated with the inhibitor of glycolysis (DOG). Furthermore, treatment with DOG also led to a dose-dependent increase on sperm susceptibility to lipid peroxidation. Based on our results we suggest that the glycolysis appears to be as important as oxidative phosphorylation for ovine sperm kinetics since this mechanism is capable of maintaining full motility when most of the cells have a low mitochondrial membrane potential. Furthermore, we found that changes in the glycolytic pathway trough glycolysis inhibition are likely involved in mitochondrial dysfunction and sperm oxidative unbalance.

3.1 INTRODUCTION

The endosymbiotic theory states that a prokaryotic organism, capable of metabolizing oxygen and producing high amounts of ATP, started living in symbiosis within an eukaryotic cell. Such theory has been proposed from observations of the similarities between the inner mitochondrial membrane (IMM) and the overall cell membrane of prokaryotic organisms (ALBERTS et al., 2008). In addition, the presence of DNA and ribosomes inside de mitochondria also support such hypothesis (JOHN; JOKHI; BARRATT, 2005). The acquisition of a more efficient mechanism of energy production allowed the subsequent rise of more complex organisms with higher energy synthesis (MARGULIS, 1970; CUMMINS, 1998; AMARAL et al., 2013). Indeed, the efficiency of producing energy by an aerobic cell depends on mitochondrial functionality. The increased demand of oxygen by the mitochondria in order to perform oxidative phosphorylation requires respiration by aerobic organisms. Such process is capable of producing about 90% of the energy required for cellular metabolism (SARASTE, 1999; COPELAND, 2002).

Over the years, studies have shown the importance of the mitochondria to sperm functionality, considered the main source of ATP for cellular homeostasis and motility (TRAVIS et al., 1998; ST. JOHN, 2002). However, the role of mitochondria on sperm metabolism has been a matter of controversy. Mukai and Okuno (2004) verified that ATP levels and flagellar beat remained constant when mice sperm mitochondrial activity is inhibited, simultaneously to the supplementation of substrates for glycolysis. However, by inhibiting glycolysis and stimulating oxidative phosphorylation, authors observed that flagellar beat and ATP levels reduced sharply. These results indicate that glycolysis has an important role in murine sperm energy production. In a similar study, Nascimento et al. (2008) performed inhibitory and stimulatory treatments for both oxidative phosphorylation and glycolysis in human sperm. Authors concluded that oxidative phosphorylation, despite contributing to ATP production, is not sufficient to sustain sperm motility, confirming that the glycolytic pathway is the primary energy source for human sperm. Moreover, studies show that incubation of human spermatozoa with inhibitors of the enzymatic complex of electron carriers led to decreased sperm motility; however, the glycolytic pathway was not considered (RUIZ-PESINI et al., 2000; JOHN; JOKHI; BARRATT, 2005).

Additionally, ATP produced by oxidative phosphorylation in the sperm midpiece is not released efficiently in distal portions of the tail, indicating that glycolysis has a key role for the flagellar beat of such sperm regions (NEVO; RIKMENSPOEL, 1970; TURNER, 2003).

Mitochondrion is essential for both sperm functionality and fertilizing capacity (MARCHETTI et al., 2002; MARCHETTI et al., 2004; GALLON et al., 2006; ST JOHN; BOWLES; AMARAL, 2006). However, it is still uncertain the actual contribution of this organelle to the overall sperm energy capacity. In fact, controversial results lead to the idea that mitochondria may have different contributions to sperm metabolism, depending on biological conditions and species involved (STOREY; BAYARD, 2008; AMARAL et al., 2013).

Despite the importance of mitochondria to sperm metabolism, metabolites also known as Reactive Oxygen Species (ROS) are produced during oxidative phosphorylation. These substances participate in many physiological reproductive processes that are dependent of oxidation (DE LAMIRANDE et al., 1997). However, an imbalance between ROS production and antioxidant mechanisms leads to the so called oxidative stress, which may be lethal to spermatozoa (HALLIWELL; GUTTERIDGE, 1999; NICHI et al., 2007). Because mitochondria is the main source of pro-oxidative factors, it is suggested, therefore, that this organelle has a central role in oxidative imbalance (AGARWAL et al., 2014). Sperm is particularly susceptible to oxidative stress due to the extremely reduced cytoplasm content, which renders a limited cytoplasmic antioxidant capacity. In addition, the high amount of polyunsaturated fatty acids in the sperm membrane, although providing membrane fluidity, is more easily oxidized. Hence, different sperm structures are impaired during oxidative stress, leading to decreased sperm quality (VERNET; AITKEN; DREVET, 2004; NICHI et al., 2007; AGARWAL et al., 2014).

Despite the information from several species regarding the role of oxidative phosphorylation and glycolysis on sperm functionality, such knowledge for ram sperm is still lacking. Studies aiming to assess sperm energetic metabolism may contribute to the understanding of the possible causes under decreased sperm quality, not only for ruminants but even for human sperm. Therefore, in this study we evaluated ram sperm functionality in response to mitochondrial uncoupling and glycolysis inhibition aiming to assess the importance of each pathway.

3.2 MATERIAL AND METHODS

The present experiment was conducted according to ethical guidelines for animal experiments and was approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science – University of São Paulo (protocol number 7978040914). Unless otherwise stated, all chemicals utilized in this study were purchased from Sigma Chemical® (St. Louis, MO, USA).

3.2.1 Animals and experimental design

Ejaculates were collected twice from six healthy and sexually mature rams by means of an artificial vagina in a weekly interval. Minimal sperm motility considered as an inclusion criteria was 60%. Therefore, only 10 ejaculates were utilized.

Immediately after collection, samples were maintained in water bath at 37°C for subsequent treatments and analysis. Sperm was diluted in modified TALP (PARRISH et al., 1988), without the presence of substrates for both glycolysis and oxidative phosphorylation, to a final concentration of $200x10^6$ spermatozoa / ml. The diluted semen was then divided into aliquots of 1000µL in such a way as to consider 7 experimental groups: Control Group (untreated sperm samples), CCCP Groups (sperm samples treated with different concentration of the oxidative phosphorylation uncoupler carbonyl cyanide meta-chlorophenyl hydrazine – CCCP; CCCP I - 20µM, CCCP II - 40µM and CCCP III - 80µM) and DOG Groups (sperm samples treated with different concentration of the glycolysis inhibitor 2-deoxy-D-glucose - DOG; competitive glucose analogue, DOG I - 5mM, DOG II - 10mM e DOG III - 50µM). Treated groups were incubated in a water bath at 37 ° C for 30 min and then subjected to sperm analysis.

3.2.2 Sperm analysis

After incubation, samples were evaluated for computer assisted sperm analysis and the following functional tests: integrity of sperm plasma and acrosomal membranes, sperm mitochondrial activity, sperm mitochondrial membrane potential (MMP) and susceptibility to lipid peroxidation.

3.2.3 Computer assisted sperm analysis

Sperm movement patterns were assessed using the Computer Assisted Sperm Analysis (CASA; Hamilton-Thorne, Ivos 12.3, USA). The following variables were considered: motility (%), progressive motility (%), VAP (average path velocity, µm/s), VSL (straight-line velocity, µm/s), VCL (curvilinear velocity, µm/s) ALH (amplitude of lateral head displacement, µm), BCF (beat cross-frequency, Hz) STR (straightness, %) and LIN (linearity, %). In addition to these parameters, the sperm velocity were also divided into four groups: rapid (VAP> 50µm / s,%), medium (30µm / s <VAP <50µm / s; %), slow (VAP <30µm / s or VSL <15µm / s;%) and static (%)(GOOVAERTS et al., 2006).

3.2.4 Sperm Functional tests

Plasma membrane integrity was assessed by eosin – nigrosine staining (Barth and Oko 1989). To perform the technique, 5µL of eosin–nigrosin stain was mixed with 5µl of semen on a microscope slide and subsequently smeared. The slides were analyzed in a conventional microscope (Nikon® E200, Tokyo, Japan) at 1000x magnification under oil immersion. One hundred cells were counted, classified as intact and injured membranes.

Sperm acrosomal integrity was assessed using the fast-green / bengal-rose staining (POPE; ZHANG; DRESSER, 1991) adapted for rams, which was performed

by mixing 5μL of the stain with 5 μl of semen on a microscope slide. After 60 seconds, this mixture was smeared and one hundred cells were counted in a conventional microscope at 100x magnification and classified as sperm showing intact or damaged acrosomes.

We assessed sperm mitochondrial activity by means of a cytochemical technique with 3'3 diaminobenzidine stain (DAB assay), which is oxidized by the cytochrome c enzyme and forms a brown colored complex that is deposited on active mitochondria (HRUDKA, 1987). Briefly, 20μL of semen was incubated with 20μL of 3’3 diaminobenzidine in an amber microcentrifuge tube for 1 hour in water bath at 37 ° C. After incubation, the mixture was smeared on microscopy slides in dark ambient. Slides were subsequently fixed in 10% formaldehyde for 10 minutes. Analysis was performed in phase contrast microscopy at 1000 x magnification under immersion oil. One hundred cells were counted and classified into 4 classes according to the percentage of stained midpiece: completely stained, indicating high mitochondrial activity (DAB I); more than 50% of the midpiece stained, indicating medium activity (DAB II); less than 50% of the mid-piece stained, indicating low activity (DAB III); and midpiece completely unstained, indicating absence of mitochondrial activity (DAB IV).

To assess mitochondrial membrane potential (MMP), we used the fluorescent probe JC-1 (iodide 5,5 ', 6,6' tetrachloro 1,1,3,3 'tetraetilbenzimidazolilcarbocianine) that was performed in flow cytometry (Guava EasyCyteTM Mini System, Guava® Technologies, USA), according to the methodology used by Hamilton et al. (2016). This equipment contains a blue laser that operates at 488nm and emits a 20mW laser radiation. To perform the technique, 187,500 spermatozoa diluted in 12.5μL TALP medium, were added to 0,5μL of the fluorescent probe JC-1 (76.5mM) and incubated at 37 ° C for 5 minutes. A total of 10,000 events per sample were analyzed, and data corresponding to yellow (PM1 photodetector, 583 nm) were recorded after logarithmic amplification. The analyses were performed using Flowjo® version 8.7 software (Ashland, OR, USA). Samples were classified into the percentages of sperm with high (JC-1 high), intermediate (JC-1 intermediate) and low (JC-1 low) mitochondrial membrane potential.

Sperm susceptibility to lipid peroxidation was assessed by the TBARS assay (Thiobarbituric Acid Reactive Substances) according to the methodology adapted by Nichi et al. (2006). Initially, samples were submitted to induction of lipid peroxidation through the incubation of 200μl of semen with 50μL of ascorbic acid (20mM) and

50µL of iron sulfate (4mM) in water bath at 37°C for 90 minutes. After induction, ice cold trichloroacetic acid 10%.(600µL) was added. Samples were then centrifuged at 20800G for 15 minutes (5°C) for precipitation of proteins and debris. Subsequently, 800µL of the supernatant were recovered and transferred to cryotubes. Thiobarbituric acid 1% (TBA; 800µL) was added and then incubated at 95° C in a water bath for 15 minutes. In this reaction, malondialdehyde (MDA; primary product of lipid peroxidation) and TBA react producing a pinkish color complex, which is quantified by spectrophotometry (Ultrospec 3300 Pro ® Amersham Biosciences, USA) at a wavelength of 532 nanometers. The susceptibility to lipid peroxidation was expressed in nanograms of TBARS / 10^6 spermatozoa.

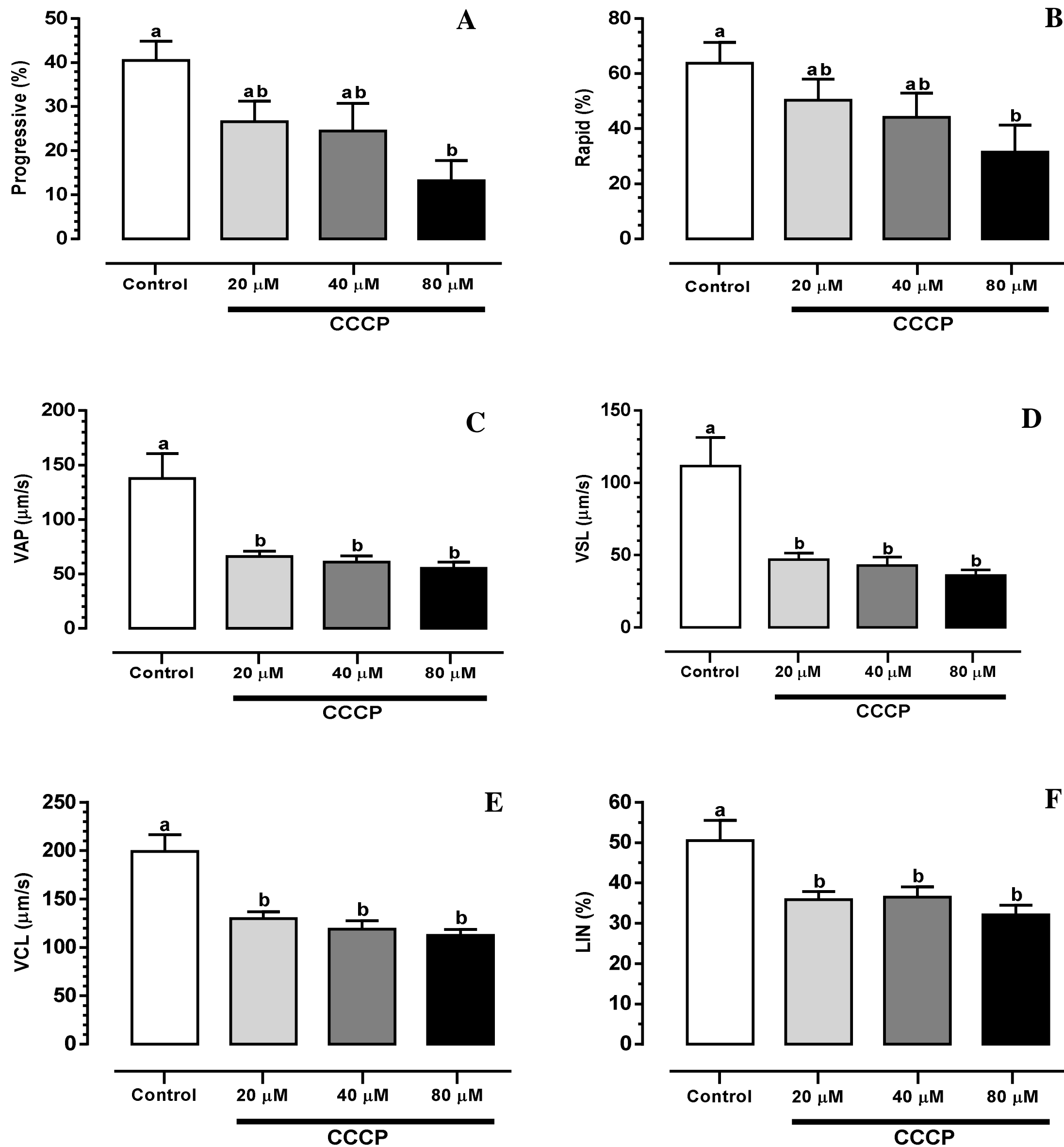
3.2.5 Statistical analysis

All data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The effect of treatments (DOG and CCCP) was determined using parametric (LSD test) and non-parametric (Wilcoxon) tests, according to the residue normality (Gaussian distribution) and variance homogeneity of each variable. A probability value of $p < 0.05$ was considered statistically significant. Results are reported as untransformed means ± S.E.M.

3.3 RESULTS

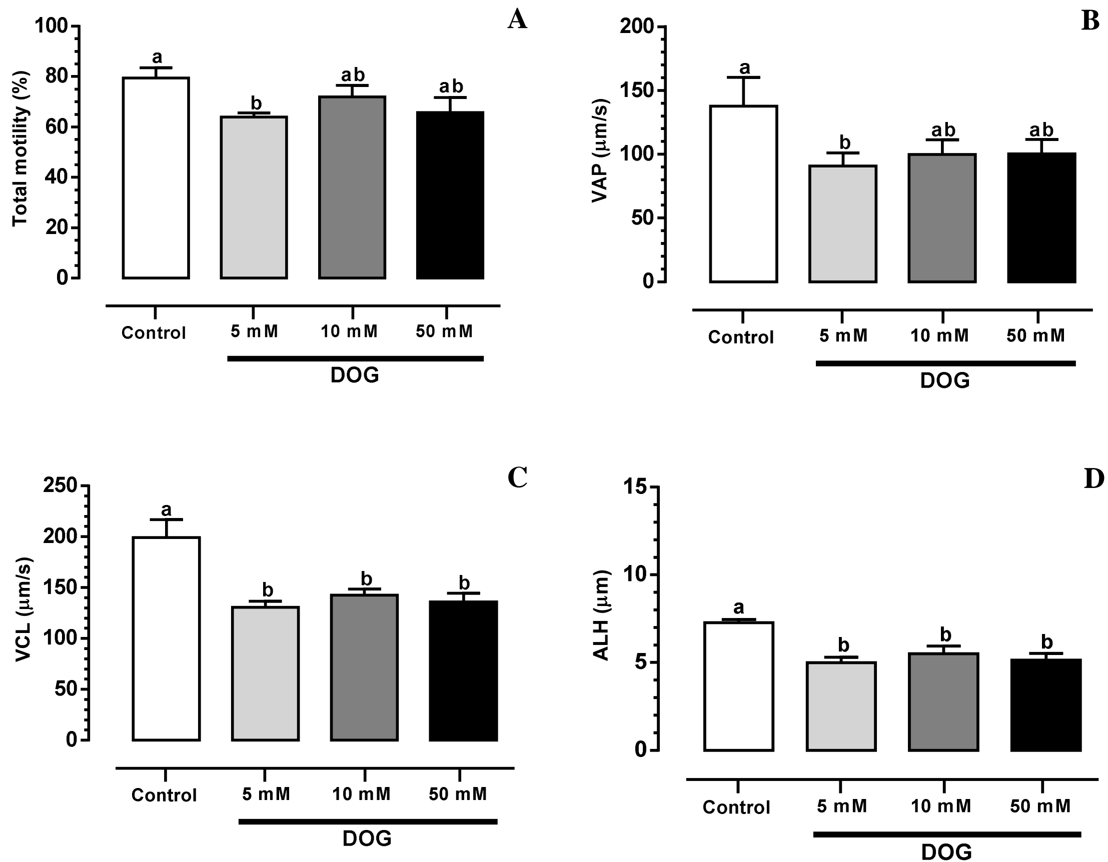
No effect of CCCP on total motility occurred, however, we verified significant alterations on sperm kinetics (FIGURES 1A-F). Samples treated with CCCP showed lower average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL) and linearity (LIN) when compared to the control group. In addition, samples treated with 80mM of CCCP (CCCP III) had decreased percentage of cells with progressive motility and rapid velocity when compared to the control group.

Figure 1- Effect of CCCP treatment (20, 40 and 80µm) on sperm kinetic parameters: progressive motility (A), spermatozoa with rapid movement (B), VAP (C), VSL (D), VCL (E) and linearity (F). Different letters indicate statistical difference between treatments ($p < 0.05$) – São Paulo - 2016



Similarly to CCCP groups, treatment with the inhibitor of glycolysis also changed sperm movement patterns (FIGURES 2A-D). DOG treated groups had higher curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) compared to the control group. In addition, the group treated with the lowest concentration of DOG presented lower average path velocity (VAP), total motility and percentage of static cells compared to the control group.

Figure 2 - Effect of DOG treatment (5, 10 and 50mM) on sperm kinetic parameters: total motility (A), VAP (B), VCL (C) and ALH (D). Different letters indicate statistical difference between treatments (p <0.05) – São Paulo – 2016



With regards to the sperm functional characteristics, we observed a lower percentage of cells with intermediate sperm mitochondrial activity in samples treated with the highest concentration of CCCP (Figure 3 A), in addition to a decrease in the percentage of cells with low sperm mitochondrial activity (Figure 3 B) and high sperm mitochondrial membrane potential (Figure 4A) in comparison to the control group. Additionally, the highest CCCP concentration promoted a decrease on sperm susceptibility to lipid peroxidation (Figure 5A).

Incubation with 50mM of DOG (DOG III) led to a decrease on the percentage of cells with low sperm mitochondrial activity (Figure 3C) and low sperm mitochondrial membrane potential (Figure 4B), comparing to the control group. On the other hand, treatment with DOG induced a dose-dependent increase on sperm susceptibility to lipid peroxidation (Figure 5C).

Figure 3 - Effect of CCCP treatment (20, 40 and 80µm) on the percentage of cells with medium and low mitochondrial activity (DAB II and DAB III, figures A and B respectively); effect of DOG treatment (5, 10 and 50mM) on the percentage of cells with low mitochondrial activity (DAB III; figure C). Different letters indicate statistical difference between treatments (p <0.05) – São Paulo - 2016

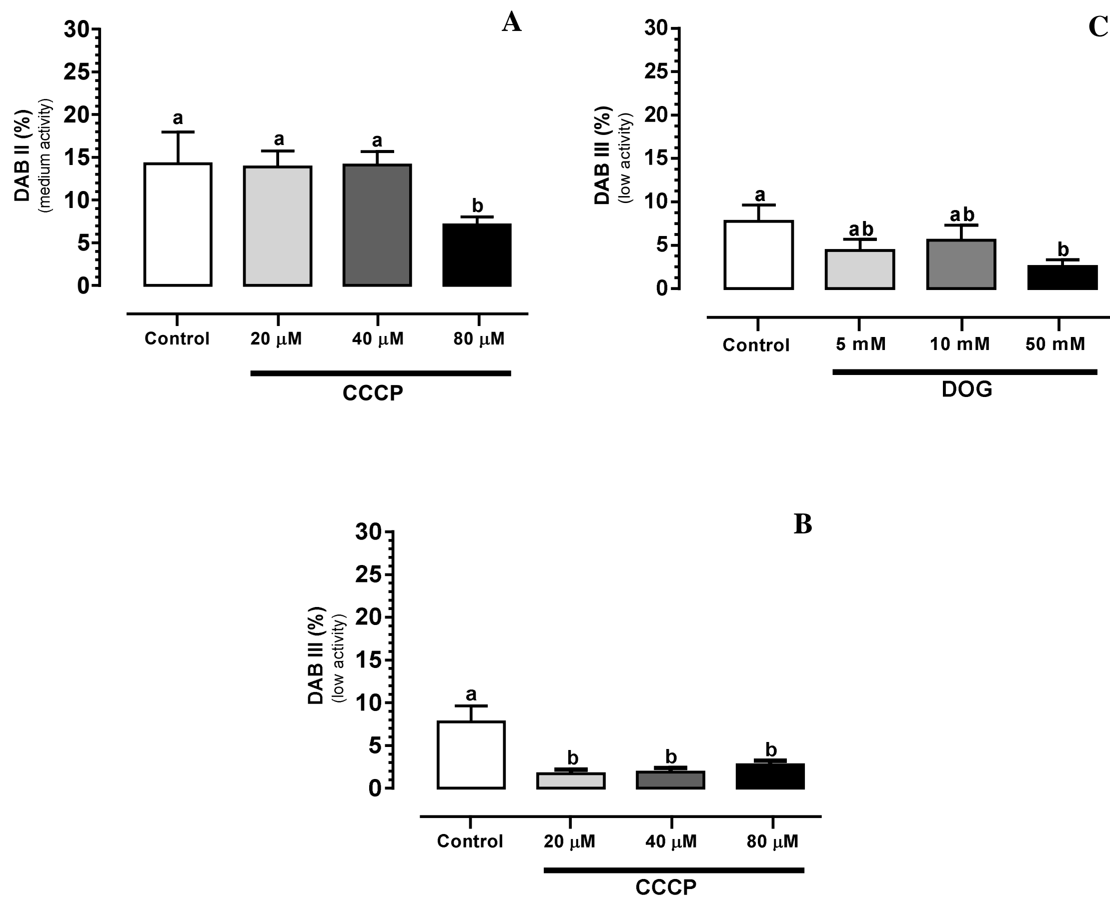


Figure 4- Effect of different concentrations of CCCP (20, 40 and 80µm; A) and DOG (5, 10 and 50mM; B), in the percentage of cells with high and low mitochondrial membrane potential respectively (high and low MMP). Different letters indicate statistical difference between treatments ($p < 0.05$). Figure 2C and 2D illustrates the histogram representing the JC1 analysis of the CCCP (80µm) and DOG (50 mM) effect compared to the control group in the populations of cells with low (L), intermediate (I) and high (H) mitochondrial membrane potential – São Paulo – 2016

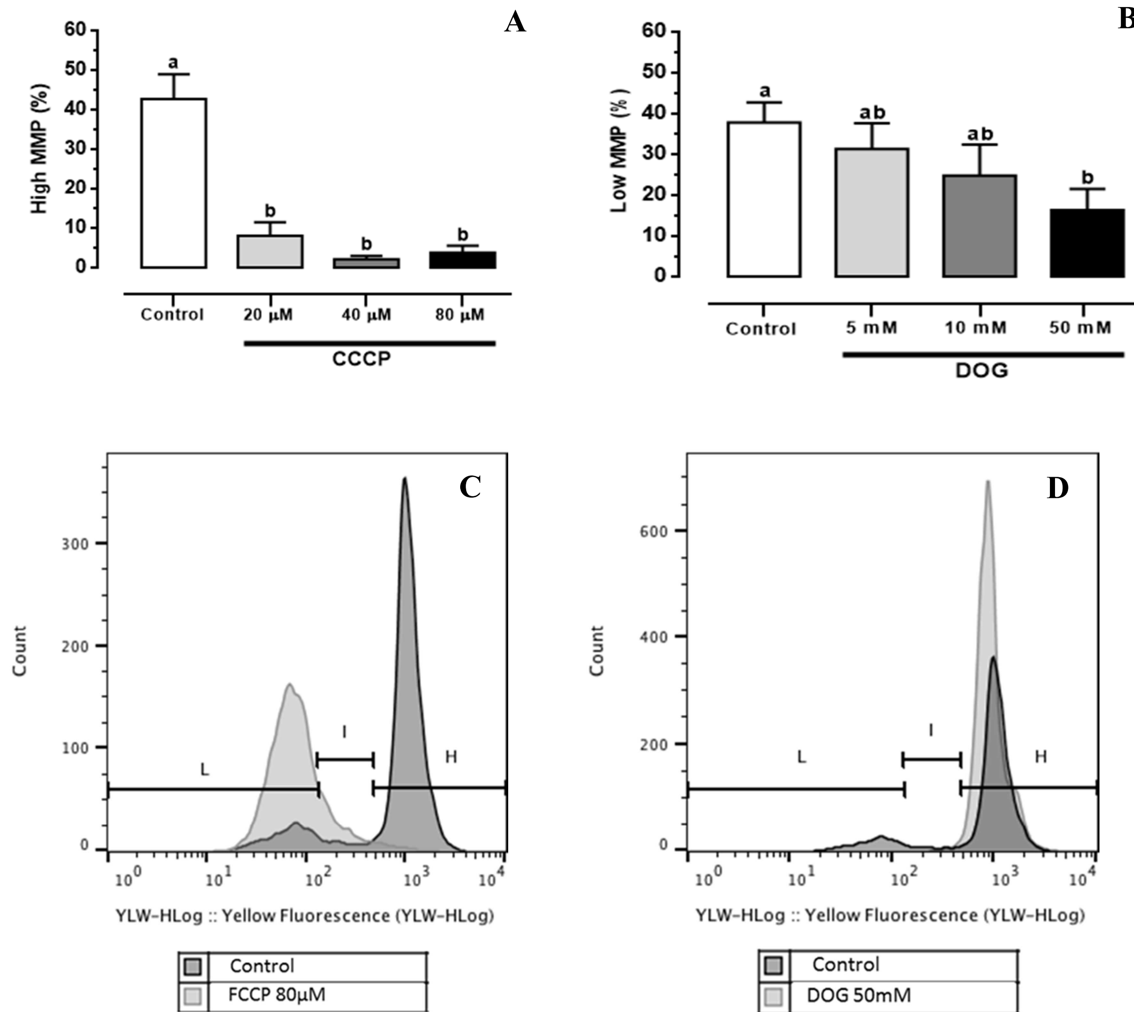
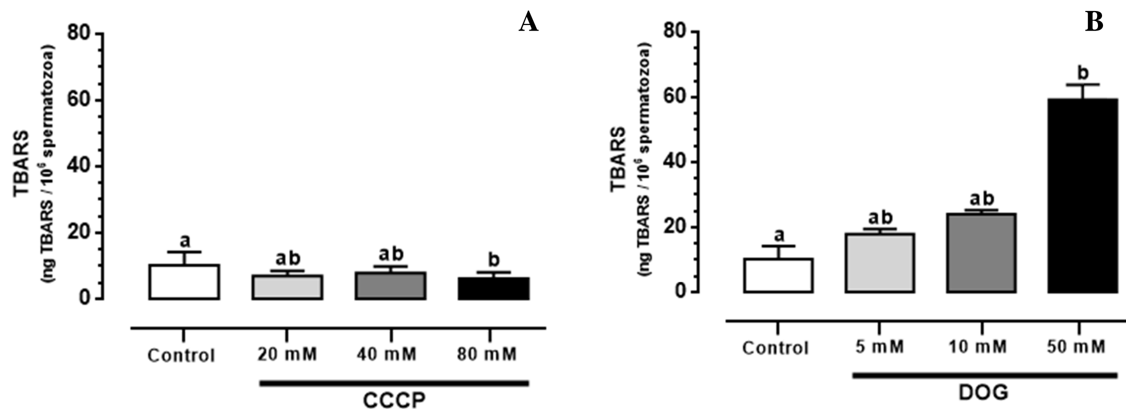


Figure 5 - Effect of CCCP (20, 40 and 80µm; figure A) and DOG (5, 10 and 50µM; figure B) treatments on lipid peroxidation (expressed in nanograms of TBARS per mL). Different letters indicate statistical difference between treatments (p <0.05) – São Paulo - 2016



3.4 DISCUSSION

The role of mitochondria as an essential source of ATP for sperm functionality is still controversial (TRAVIS et al., 1998; ST. JOHN, 2002; MUKAI; OKUNO, 2004). For some species, the glycolytic pathway has been suggested to be even more important for sperm motility than oxidative phosphorylation (MUKAI; OKUNO, 2004; NASCIMENTO et al., 2008). Therefore, the knowledge regarding the importance of sperm energetic balance mechanisms is lacking for several animal species including the ovine. Thus, we designed the present study by uncoupling or inhibiting oxidative phosphorylation and glycolysis of ovine sperm in order to evaluate the influence of such pathways on the movement patterns and functional characteristics.

ATP synthesis in the mitochondria occurs through the coupling of two reactions: the transport of electrons throughout the respiratory chain and the proton gradient. This latest gradient is capable of storing energy, called proton motrice force, which drives the synthesis of ATP through ADP and inorganic phosphate (LOWELL; SHULMAN, 2005). The mitochondrial uncoupler CCCP is a lipophilic molecule with protonophore properties, in other words, it is capable of interacting with the inner mitochondrial membrane allowing pumped protons to return to the mitochondrial matrix, dissipating the proton gradient and influencing the mitochondrial chemiosmosis. The proton gradient dispersion can interfere with the ATP synthesis.

However, CCCP does not have a direct effect on the enzyme ATP synthase, the electron transport chain and neither on the Krebs cycle (TERADA, 1990). In our study, we used high concentrations of CCCP aiming to significantly reduce mitochondrial function and, therefore reducing ATP synthesis.

Mitochondrial activity and mitochondrial membrane potential (MMP) are parameters that, although related, should be considered separately. While mitochondrial activity refers to electrons transport through the respiratory chain, MMP concerns to the difference in H^+ concentrations between the intermembrane space and the mitochondrial matrix. The cytochemical assay diaminobenzidine (DAB assay) is used to assess mitochondrial activity by measuring the efficiency of the cytochrome C enzyme to transport electrons in the respiratory chain complex IV to molecular oxygen (HRUDKA, 1987). On the other hand, JC-1 is a lipophilic metachromatic probe that easily penetrates the mitochondria identifying cell populations with different MMP (i.e., different concentrations of protons between the intermembrane space and mitochondrial matrix) (CHEN, 1988; REERS; SMITH; CHEN, 1991). Depending on the concentration, mitochondrial uncouplers have the ability to decrease mitochondrial membrane potential. However, while the MMP decreases, there is an increase in electron transport rates between the mitochondrial complexes (CALDEIRA DA SILVA et al., 2008). This is due to a compensatory mechanism in which mitochondrial complex pump more protons to the intermembrane space in an attempt to reestablish the MMP. In fact, we verified a decrease in the percentage of sperm with highly impaired mitochondrial activity (DAB III) in groups treated with CCCP, indicating an improvement on energy transport rates. Conversely, we show a drastic increase in the percentage of sperm with low MMP in CCCP treated groups, which confirms the previously mentioned mechanism.

Another interesting point of mitochondrial activity refers to its impact on sperm functionality and oxidative status. Studies using diaminobenzidine cytochemical assay have demonstrated that highly impaired mitochondrial activity (DABIII) rather than no activity at all (DAB IV) is involved in increased levels of oxidative stress and DNA fragmentation (BLUMER et al., 2008; BLUMER et al., 2012). The decrease in the percentage of sperm with low mitochondrial activity verified in CCCP groups suggests a possible mitochondrial protective effect. It has been demonstrated in several cell types, including spermatozoa, that mild mitochondrial uncoupling can lead to the reduction in ROS production by increasing the respiratory rate. As a

consequence, oxygen tension in the mitochondrial microenvironment is reduced, without considerably reducing ATP levels (KOSHIMOTO; GAMLIEL; MAZUR, 2000; BRENNAN et al., 2006; CALDEIRA DA SILVA et al., 2008; FANG et al., 2014). Indeed, we found that the highest concentration of CCCP promoted decreased susceptibility to lipid peroxidation, simultaneously to the decrease in the percentage of sperm with low mitochondrial activity. However, further detailed studies should be carried out in order to verify the possible protective effect of CCCP.

The fact that CCCP significantly increased the percentage of sperm with low MMP demonstrates that the mitochondrial uncoupler was effective in performing the protonophore action, depolarizing the mitochondrial membrane. Although we did not measure the levels of ATP, this can be indirectly stated by considering the protons gradient. Thus, we can assume that sperm samples treated with CCCP synthetize less mitochondrial ATP. Surprisingly, sperm motility was not impaired, although a high percentage of sperm showed low mitochondrial membrane potential. However, our results were consistent with previous study in boars, which demonstrated that sperm mitochondria accounts for only 5% of energy production while the glycolytic pathway contributes to 95% (MARIN et al., 2003). Additionally, species such as mice may use ATP from glycolysis and mitochondrial respiration depending on their biological conditions without changing sperm functionality or sperm ATP levels (PASUPULETI, 2007). Moreover, Ramió-Lluch et al. (2014) demonstrated that the inhibition of ATP synthase impairs sperm motility, while intracellular ATP levels remains unchanged. Therefore, an unknown essential mitochondrial mechanism responsible for motility maintenance which does not rely only on ATP levels should exist. Hence, such mechanism should be further studied and elucidated.

We also observed that, despite the decrease on mitochondrial membrane potential (MMP), mitochondrial uncoupling had no influence on total motility. On the other hand, sperm movement patterns such as progressive motility and percentage of sperm with rapid movement were altered in the groups treated with the uncoupler CCCP. However, motility is not the only important parameter to ensure fertility, besides lacking reference values for kinetics parameters evaluated by computer assisted analysis. On the other hand, when sperm motility is impaired, negative impact on fertility should is expected (FARRELL et al., 1998; LARSEN et al., 2000; VERSTEGEN; IGUER-OUADA; ONCLIN, 2002). Thus, we can suggest that

mitochondria has an utmost role in regulating sperm movement patterns rather than maintaining total motility as previously suggested.

The inhibition of the glycolytic pathway precludes the formation of its final product, pyruvate, which is essential for the Krebs cycle. This would indirectly affect oxidative phosphorylation. In this context, the decrease in pyruvate concentration can negatively impact electrons transport and, ultimately, the MMP (BAGKOS; KOUFOPOULOS; PIPERI, 2014). Therefore, in our work, we expected to observe MMP impairment when using the inhibitor of glycolysis (DOG). Interestingly, we found that the highest concentration of DOG (III) decreased the percentage of cells with low MMP. In fact, recent studies have demonstrated that when oxidative phosphorylation is impaired there is a compensatory mechanism that makes the ATP synthase to operate in a reverse mode. Thus, ATP would be consumed rather than produced, directing protons to the intermembrane space in order to restore MMP (BAGKOS; KOUFOPOULOS; PIPERI, 2014). Thus, our results on the higher percentage of sperm with low MMP can be attributed to a compensatory mechanism that occurred due to the impairment of oxidative phosphorylation, caused by the inhibition of the glycolytic pathway.

In our study, we show a higher sperm susceptibility to lipid peroxidation while increasing DOG concentrations. DOG is a competitive inhibitor of hexokinase, which converts glucose into glucose-6-phosphate. By inhibiting glucose 6-phosphate, not only the glycolytic pathway will be impaired but also the pentose phosphate pathway (PPP) (COLEMAN et al., 2008). This latter pathway has an essential role in preventing oxidative stress and lipid peroxidation, by producing NADPH and ribose - 5 - phosphate (KRUGER; VON SCHAEWEN, 2003; PERL et al., 2011). NADPH has a reduction action on the glutathione–peroxidase / glutathione-reductase antioxidant system (STOREY; ALVAREZ; THOMPSON, 1998), in which reduced glutathione (GSH) is used as a substrate to degrade hydrogen peroxide (H_2O_2) into water (H_2O) and oxidized glutathione (GSSG). In turns, GSSG is reduced from the glutathione reductase enzyme (GRD), dependent on the conversion of NADPH to NADP (NORDBERG; ARNÉR, 2001). In fact, Williams and Ford (2004) demonstrated that during moderate seminal oxidative stress, the PPP pathway can respond dynamically by increasing NADPH and, consequently, activating the glutathione-peroxidase/glutathione – reductase system. Thus, authors suggest a possible modulating capacity of PPP to maintain oxidative homeostasis. Therefore, the

indirect inhibition of PPP can lead to an increase in lipid peroxidation, which, in fact, was observed in the preset study. However, further studies should be conducted using specific inhibitors of PPP to attest its role on cellular oxidative homeostasis. Additionally, dysfunctions of the glycolytic pathway are known to be associated with several diseases such as diabetes (ROBERTSON, 2004; NISHIKAWA; ARAKI, 2007), coronary heart disease (LEYVA et al. 1998) and cancer (CAIRNS; HARRIS; MAK, 2011). In our experiment, pyruvate synthesis impairment due to the glycolytic pathway inhibition can also be one of the factors under the increase on lipid peroxidation, since pyruvate is essential for mitochondrial respiration. Indeed, studies have shown the importance of pyruvate on sperm oxidative homeostasis and its protective role in oxidative processes (QIU et al. 2016; BILODEAU et al., 2002; FERRAMOSCA et al., 2016). However, it is yet to be elucidated the effect of the glycolytic pathway disorder or inhibition on oxidation process.

Similarly to the results observed for CCCP-treated samples, DOG promoted significant changes on sperm movement patterns. Despite the fact that glycolysis is energetically less efficient than oxidative phosphorylation, we observed that this metabolic pathway seems to be extremely important for sperm kinetics. In spite of the uncertain role of glycolysis on sperm physiology, studies have increasingly shown its importance for sperm functionality in a number of species (WHITE; WALES, 1961; KRZYZOSIAK; MOLAN; VISHWANATH, 1999; MUKAI; OKUNO, 2004; NASCIMENTO et al., 2008). Additionally, studies suggest that molecules of ATP, produced by oxidative phosphorylation in the sperm intermediary piece, are not spread efficiently towards more distal portions of the tail. In such sperm regions glycolysis may have a fundamental influence to maintain flagellar beat (NEVO; RIKMENSPOEL, 1970; TURNER, 2003). Therefore, glycolysis inhibition may have altered sperm kinetics by reducing ATP availability in the more distal parts of the flagellum.

In conclusion, the glycolytic pathway appears to be as important as oxidative phosphorylation for ovine sperm kinetics since this mechanism is capable of maintaining full motility in spite of a low MMP. Furthermore, despite the central role of mitochondria on sperm oxidative balance, glycolysis inhibition seems to influence cellular oxidative homeostasis. Therefore, changes in glycolysis are likely to be involved in sperm oxidative homeostasis.

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Chapter 2

4 CHAPTER 2: The stimulated glycolytic pathway is able to maintain ATP levels and kinetic patterns of bovine epididymal sperm submitted to mitochondrial uncoupling

ABSTRACT

Studies have reported the importance of mitochondria in sperm functionality. However, for some species, the glycolytic pathway appears to be as important as oxidative phosphorylation in ATP synthesis and sperm kinetics. These mechanisms have not been fully elucidated for bovine spermatozoa. Therefore, the aim of this study was to evaluate the role of mitochondria and the glycolytic pathway in ATP synthesis, sperm movement patterns and oxidative homeostasis of epididymal spermatozoa in bovine. We observed that mitochondrial uncoupling significantly reduced ATP levels. However, these levels were re-established after stimulation of the glycolytic pathway. We verified the same pattern of results for sperm kinetic variables and the production of reactive oxygen species (ROS). Thus, we suggest that the glycolytic pathway, after stimulation, is capable of maintaining ATP levels, sperm kinetic patterns and oxidative balance of bovine epididymal spermatozoa submitted to mitochondrial uncoupling.

4.1 INTRODUCTION

Studies have shown the importance of mitochondria in sperm functionality, as they are considered the main source of ATP for cellular homeostasis and motility (TRAVIS et al., 1998; ST. JOHN, 2002). However, the role of mitochondria in sperm metabolism has been a matter of debate. Mukai and Okuno (2004) verified that ATP levels and flagellar beating remained constant when the mitochondria of mouse

sperm was uncoupled concurrently with glycolysis stimulation. However, by inhibiting glycolysis and stimulating oxidative phosphorylation, authors observed that flagellar beating and ATP levels quickly reduced. These results indicate that glycolysis plays an important role in murine sperm energy production.

In a similar study, Nascimento et al. (2008) performed inhibitory and stimulatory treatments for both oxidative phosphorylation and glycolysis in human sperm. Authors concluded that oxidative phosphorylation, despite contributing to ATP production, is not sufficient to sustain sperm motility, confirming that the glycolytic pathway is the primary energy source for human sperm. Additionally, ATP produced by oxidative phosphorylation in the sperm midpiece is not efficiently released into the distal portions of the tail, indicating that glycolysis plays a key role in the flagellar beat of such sperm regions (NEVO; RIKMENSPOEL, 1970; TURNER, 2003; DU PLESSIS et al., 2015).

The role of mitochondria and the glycolytic pathway for bovine sperm has not been fully elucidated. This information is extremely important for the understanding of bull sperm physiology. In addition, studies evaluating the energy metabolism of bovine sperm may contribute to the understanding of possible causes for the reduction in sperm quality and fertilization failures related to these metabolic pathways.

Sperm collected directly from the epididymis seem to be the ideal cellular model to study energy metabolism. This is due to the many glycolysis, citric acid cycle and oxidative phosphorylation stimulants contained in the seminal plasma derived from the accessory glands (GARNER; HAFEZ, 2000; ZÖPFGEN et al., 2000; AGUIAR et al., 2013). The fact that epididymal spermatozoa have not been stimulated with these substances provides a better in vitro manipulation of these cells, allowing the stimulation and inhibition of these pathways to evaluate the role of each metabolic pathway on sperm functionality.

Therefore, the aim of this study was to evaluate the role of mitochondria and glycolysis in ATP production, generation of reactive oxygen species (ROS) and kinetic patterns of epididymal bovine sperm by means of mitochondrial uncoupling and glycolytic pathway stimulation.

4.2 MATERIAL AND METHODS

The present experiment was conducted according to ethical guidelines for animal experiments and approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science at the University of São Paulo (protocol number 7978040914).

In this study, we submitted bovine epididymal spermatozoa to treatment with the oxidative phosphorylation uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) to significantly reduce mitochondrial ATP synthesis and stimulated the glycolytic pathway by glucose addition. However, in order to verify the optimal concentrations of the uncoupler, FCCP, we performed a dose-response curve in experiment 1. Thus, the selected concentrations were used in the subsequent experiments. The aim of these experiments was to evaluate the contribution of mitochondria to ATP synthesis (experiment 2), patterns of sperm kinetics (experiment 3) and oxidative homeostasis (experiment 4) of bovine epididymal sperm and verify if stimulation of the glycolytic pathway would be able to maintain these sperm parameters that are probably suppressed by mitochondrial uncoupling.

4.2.1 Experiment 1 - dose-response curve of mitochondrial uncoupler, FCCP

To accomplish the dose-response curve, sperm were collected from three bovine epididymides. The samples were diluted in modified TALP to a final concentration of 100 million spermatozoa per mL. Thereafter, the spermatozoa were incubated in a perfusion chamber with mitochondrial fluorophore tetramethylrhodamine-ethyl-ester perchlorate at 500nM (ThermoFisher® Scientific , 0.5μL of TMRE in 1 mL of medium) for 5 minutes at 37°C. For the spermatozoa to remain attached during perfusion with FCCP, coverslips of the perfusion chamber were treated with polylysine.

After incubation, the amount of TMRE fluorescence captured by each sperm mitochondria was recorded by the software LAS AF Lite (Leica® Microsystems, Germany) at an emission of 500 nm and excitation of 600 nm by microscope using X (Leica® Microsystems, Germany). Thirty seconds of mitochondrial basal fluorescence was recorded, and then perfusions were performed with increasing FCCP concentrations (Tocris Bioscience®, MN, USA; 0.3, 1, 3, 10, 30, 60 and 100μM) by means of an electrovalves controller. Stimulation performed with FCCP at 30 seconds was recorded, and the percentage of mitochondrial depolarization was calculated based on the difference between the basal fluorescence and the amount of fluorescence retained in the mitochondria of each spermatozoa after 30 seconds of FCCP stimulation.

The lower FCCP concentrations of the dose-response curve (0.3, 1, and 3μM) and the concentration insufficient for the promotion of mitochondrial depolarization (0.1μM, concentration under the curve) were selected for use in the subsequent experiments. We selected these concentrations in order to significantly reduce the mitochondrial ATP synthesis without promoting disruption in this organelle.

4.2.2 Experiment 2 - Effect of mitochondrial uncoupling and glycolysis stimulation on ATP levels

In this experiment, spermatozoa from 6 bovine epididymides (n = 6) were collected and diluted to a concentration of 100 million spermatozoa per mL in modified TALP. Each sample was divided into ten aliquots, which were submitted to a 5 x 2 factorial design wherein one of the factors was the addition of glucose (5mM) and the other factor was the treatment with increasing concentrations of FCCP (0.1, 0.3, 1 and 3μM). After a 15-minute incubation, the treatments were subjected to measurements of ATP levels by means of a luminescence technique. For this procedure, 50μL aliquots in duplicate from each treatment containing 100,000 spermatozoa were added to 50μL of CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega®, USA) and incubated for 30 minutes at 37 ° C according to the manufacturer's recommendations. Immediately after this procedure, ATP levels were

measured in a luminescence apparatus (ThermoFisher® Scientific, MA, USA) in duplicate. The results obtained, expressed in arbitrary light units (AUL), were interpolated on a standard curve containing different concentrations of ATP (10, 100, 1000, 5000 and 10000nM) and were then expressed in nM ATP.

4.2.3 Experiment 3 – Effect of mitochondrial uncoupling and glycolysis stimulation on sperm kinetic patterns

To evaluate the effect of mitochondrial uncoupling and glycolysis stimulation on sperm kinetic patterns, spermatozoa from 7 bovine epididymides (n = 7) were collected and diluted to a concentration of 100 million spermatozoa per mL in modified TALP. Each sample was divided into ten aliquots, which were submitted to a 5 x 2 factorial design wherein one of the factors was the addition of glucose (5mM) and the other was the treatment with increasing concentrations of FCCP (0.1, 0.3, 1 and 3μM). After 5 minutes of incubation, the sperm samples were subjected to computerized analysis of sperm kinetics (ISASPBOS, Proiser®, Valencia, Spain). The following variables were considered: motility (%), progressive motility (%), VAP (average path velocity, μm/s), VSL (straight-line velocity, μm/s), VCL (curvilinear velocity, μm/s) ALH (amplitude of lateral head displacement, μm), BCF (beat cross-frequency, Hz) STR (straightness, %) and LIN (linearity, %). In addition to these parameters, the sperm were also divided into four groups based on velocity: rapid (VAP> 50μm /s; %), medium (30μm /s <VAP <50μm /s; %), slow (VAP <30μm /s or VSL <15μm /s; %) and static (%) (GOOVAERTS et al., 2006).

4.2.4 Experiment 4 - Effect of mitochondrial uncoupling and glycolysis stimulation on reactive oxygen species production

To evaluate the effect of mitochondrial uncoupling and glycolysis stimulation on reactive oxygen species production, spermatozoa from 6 bovine epididymides (n = 6) were collected and diluted to a concentration of 100 million spermatozoa per mL

in modified TALP. Each sample was divided into ten aliquots, which were submitted to a 4 x 2 factorial design wherein one of the factors was the addition of glucose (5mM) and the other was the treatment with increasing concentrations of FCCP (0.1, 0.3, 1 and 3μM). These treatments were incubated for 30 minutes at 37 ° C and subjected to the detection of reactive oxygen species. To perform this technique, 100,000 sperm were incubated in modified TALP solution containing 10μM (final concentration) of the fluorescent probe CM-H2DCFDA for 30 minutes (triplicate samples). After incubation was performed, the ROS were detected using a fluorimeter (Fluostar microplate reader Omega, Labtec-BMG, Germany) at excitation 492-495 nm and emission 517-527 nm. The fluorescence intensity results obtained were interpolated on a standard curve containing different concentrations of hydrogen peroxide (H_2O_2; 3, 10, 30, 60, 100, 200 and 300μM) and were then expressed in μL of O_2 generated. Data were normalized relative to the control group (untreated samples).

4.2.5 Statistical analysis

The dose-response curve for FCCP (Experiment 1) was performed by nonlinear regression using the statistical program GraphPad Prism 6. Data relating to the measurement of ATP levels and computerized analysis of sperm kinetics (experiments 2 and 3, respectively) were analyzed using the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Thus, the interaction between FCCP and glucose factors were determined by PROC GLM. Differences between treatments were assessed using parametric (Student's t test for each factor separately or LSD test for the combination of factors) and nonparametric tests (Wilcoxon) in accordance with the normality of the residuals (Gaussian distribution) and homogeneity of the variances. To analyze the effect of FCCP in the presence or absence of glucose in the production of ROS, data normalized to the control group were compared by ANOVA variance analysis (LSD test) using the SAS System for Windows program (SAS Institute Inc., Cary, NC, USA). The level of significance to reject the H0 (null hypothesis) was 5%; that is, the significance level was 0.05.

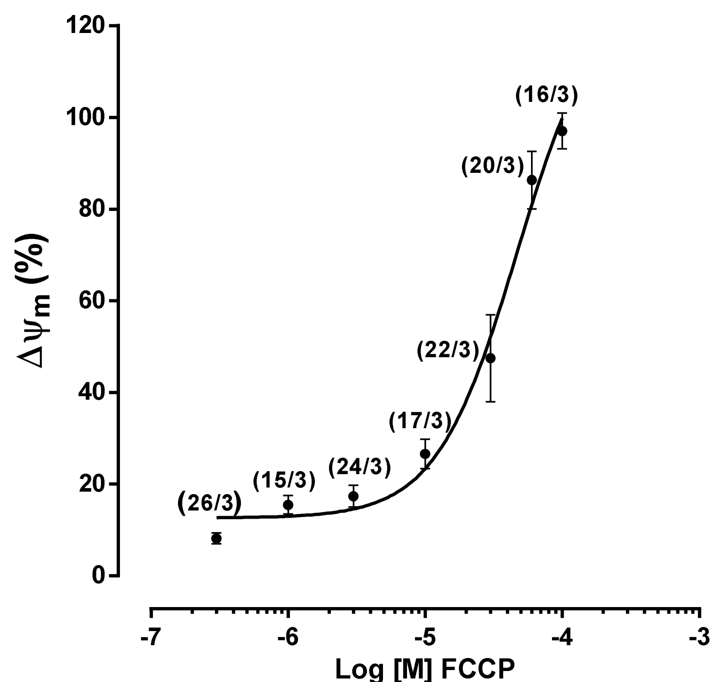
Significant differences between classificatory variables (treatments) to a specific response variable were considered.

4.3 RESULTS

4.3.1 Experiment 1 – Dose-response curve of mitochondrial uncoupler FCCP

By using a non-linear regression, we found that the dose-response curve square root = 0.7 and EC50 = 4.67 x 10^{-5} µM. We observed a high percentage of depolarization with FCCP concentrations of 30µM, 60µM and 100µM (Figure 6). Thus, in order to select points where there is a reduction in ATP without promoting disruption in the organelle, we selected 3µM, 1µM, 0.3 µM and 0.1 µM for the concentrations used in the subsequent experiments (concentration under the curve – Figure 6).

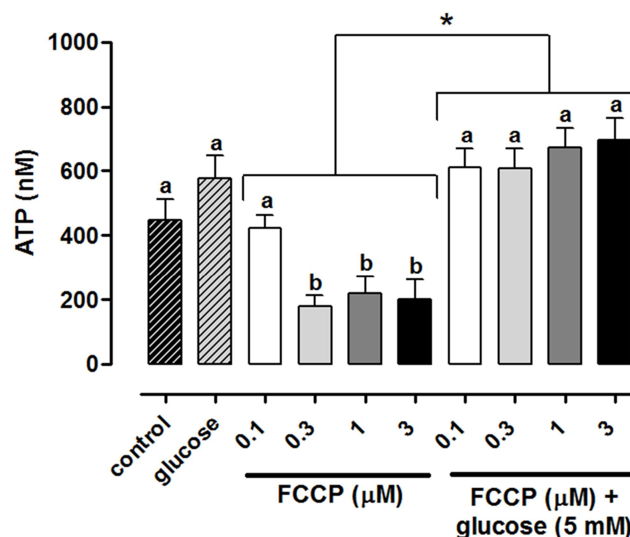
Figure 6 - Dose-response curve of FCCP concentrations (0. 3, 1, 3, 10, 30, 60 and 100µM) in sperm of bovine epididymal samples – São Paulo - 2016



4.3.2 Experiment 2 – Effect of mitochondrial uncoupling and glycolysis stimulation on ATP levels

There were significant effects of FCCP, glucose and FCCP-by-glucose interaction in the ATP (P<0.0001) analysis. Then, it was possible to compare the effects of the addition of glucose in the FCCP sample (Figure 7). We observed a lower ATP production in the FCCP group at concentrations of 0.3μM (180.3 ± 31.9nM), 1μM (220.2 ± 40.4nM) and 3μM (272.3 ± 70.4nM) than at 0μM (control – 448.6 ± 63.7nM) and 0.1 μM (422.4 ± 41.5nM – Figure 7). However, in the group treated with FCCP supplemented with glucose, the concentrations were similar between groups treated with 0.1 μM (610.8 ± 57.8nM), 0.3μM (606.2 ± 64.2nM), 1μM (670.9 ± 61.9nM), and 3 μM (696.1 ± 68.5nM) FCCP and the group treated with glucose without FCCP (577.2 ± 70.4nM) (Figure 7).

Figure 7 - ATP production by sperm treated with FCCP in different concentrations (0μM, 0.1μM, 0.3μM, 1μM and 3μM) in absence or presence of glucose 5mM– São Paulo - 2016



[a-b] Superscripts indicates differences between concentrations (P < 0.05). [*] Indicates differences after the glucose supplementation (P<0.05).

4.3.3 Experiment 3 – Effect of mitochondrial uncoupling and glycolysis stimulation on sperm kinectics patterns

There were significant effects of FCCP, glucose, and FCCP-by-glucose interaction (P < 0.05) on all CASA parameters (Table 1).

Table 1 – Probability values for the FCCP (0, 0.1, 0.3, 1 and 3μM), glucose and their interaction on computer-assisted sperm analysis (CASA) – São Paulo - 2016

	FCCP	Glucose	FCCP x Glucose
Total sperm motility (%)	<0.0001	0.0003	<0.0001
Sperm progressive motility (%)	<0.0001	0.0005	<0.0001
Percentage of rapid sperm (%)	0.0006	0.0077	<0.0001
Percentage of medium sperm (%)	0.0087	0.0033	<0.0001
Percentage of slow sperm (%)	0.3993	0.0361	0.0045
Amplitude of lateral head displacement (ALH - μm)	0.0009	0.0119	0.0095
Average path velocity (VAP - μm/s)	<0.0001	0.0002	<0.0001
Straight line velocity (VSL - μm/s)	<0.0001	0.0002	<0.0001
Curvilinear velocity (VCL - μm/s)	0.0002	0.0038	0.0002
Beat cross-frequency (BCF - Hz)	<0.0001	0.0020	<0.0001
Sperm straightness (STR - %)	0.0002	0.0020	<0.0001
Sperm linearity (LIN - %)	<0.0001	0.0003	<0.0001
Wobble (WOB - %)	<0.0001	0.0003	<0.001

We observed a decrease in the total motility between samples without FCCP (control) and with glucose (Figure 8A); however, it was possible to note an increase in motility in the groups treated with 0.3μM, 0.1μM, 1μM and 3μM FCCP supplemented with glucose (Figure 8A). This same effect was detected for progressive motility (Figure 8B), VAP, VSL, VCL and rapid sperm velocity (Table 2).

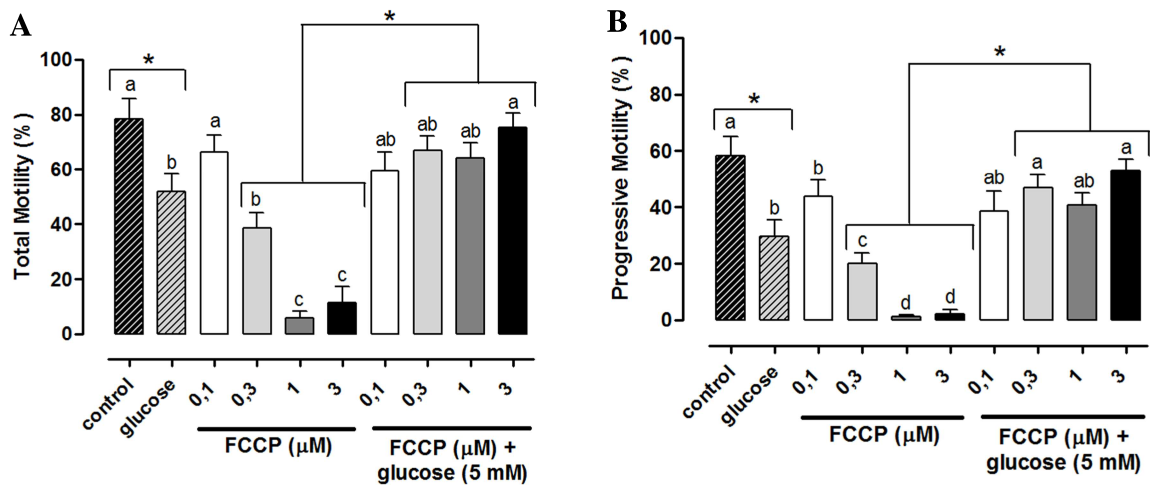
Next, we examined the effects of the addition of glucose in the FCCP samples (Figure 8 and Table 2). In the BCF analysis, we observed an increase in the groups with 1μM and 3μM of FCCP supplemented with glucose but a decrease in the glucose group (Table 2). Furthermore, we observed an increase in the slow sperm velocity in the samples supplemented with glucose in the groups treated with 1μM and 3μM of FCCP and glucose alone but a decrease in the group treated with 0.3μM FCCP (Table 2).

With FCCP treatment, the control and 0.1μM groups had higher values of total sperm motility, VAP and VSL than the 0.3μM group, which was superior to the 1μM and 3μM samples (Figure 8 and Table 2). However, in the ALH, BCF, straightness, linearity and wobble analyses, the control, 0.1μM and 3μM groups had higher rates than the 1μM and 3μM groups (Table 2). In the VCL and percentage of medium sperm velocity, we observed that the 3μM and 1μM groups had lower values than the 0.3μM group, which was similar to the 0.1μM group but lower than control (Table 2). In progressive motility (PM), the control group had the highest rates (Figure 8). However, we observed lower rates of PM in the 3μM and 1μM groups than in the 0.3μM group, which was inferior to the 0.1μM group (Figure 8). In the medium sperm velocity, the control group was superior to the 1μM and 3μM groups (Table 2). On the other hand, in the slow sperm velocity, the control and 1μM groups had lower rates than the 0.1 μM and 0.3 μM groups (Table 2).

When we compared the results between the concentrations of FCCP supplemented with glucose, we highlighted the higher values of progressive motility, straightness and rapid sperm velocity in the groups treated with 3μM and 0.3μM of FCCP, which were superior to the glucose group (Figure 8 and Table 2). In the total motility analysis, the 3μM group was superior to the glucose group (Figure 8). However, in the VCL, the 0.3μM group had higher values than the 1μM group (Table 2). The glucose group was lower than the 0.3μM, 1μM and 3μM groups in the BCF

parameter (Table 2). However, in the slow sperm velocity, the 1μM group was higher than the 0.3μM group (Table 2). The remaining CASA variables did not show any difference between groups (Table 2).

Figure 8 – Total and progressive motility in sperm treated with FCCP in different concentrations (0μM, 0.1μM, 0.3μM, 1μM and 3μM) in absence or presence of glucose 5mM – São Paulo - 2016



[a-d] Superscripts indicates differences between concentrations (P < 0.05). * Indicates differences after the glucose supplementation (P<0.05).

Table 2 – Sperm kinetics patters of sperm treated with FCCCP in different concentrations (0μM, 0.1μM, 0.3μM, 1μM and 3μM) in absence or presence of glucose 5mM – São Paulo - 2016

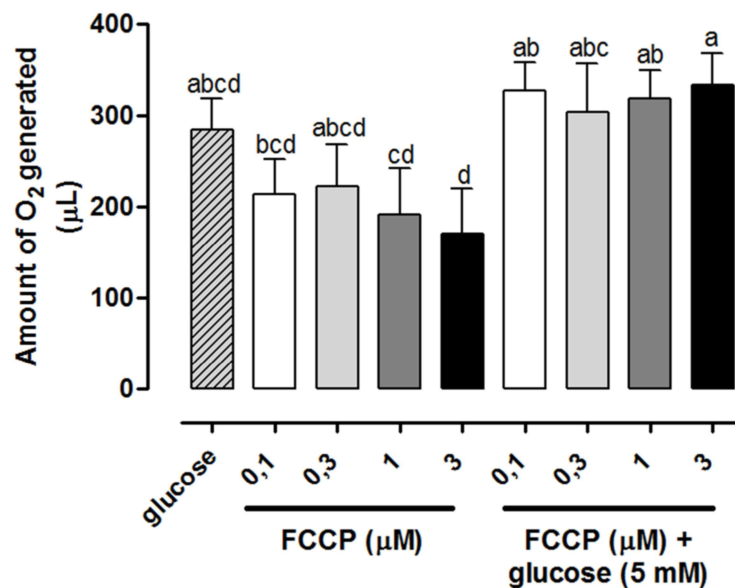
	SPERM KINETICS PATTERS									
	FCCP (μM)					**FCCP (μM) + glucose (5mM)**				
	Control	0.1μM	0.3μM	1μM	3μM	Glucose	0.1μM	0.3μM	1μM	3μM
VAP (μm/s)	50.6±3.3^{a}	41.6±2.8^{a}	30.5±3.8^{b}	7.4±2.6^{c}	8.4±3^{c}	40.9±2.5*	40±4.6	46.7±2.7*	41.6±2.8*	45.9±1.2*
VSL (μm/s)	43.4±3.3^{a}	35.6±3.6^{a}	25.4±3.5^{b}	3.6±1.6^{c}	4.9±2^{c}	32.1±2.9*	33.9±4.8	40.6±2.9*	35.7±1.9*	39.8±0.8*
VCL (μm/s)	70.6±1.6^{a}	56.7±3.1^{ab}	46.8±1.2^{b}	23.2±8.3^{c}	24.8±9.9^{c}	56.5±2^{ab*}	57.3±4.1^{ab}	63.7±2.6^{a*}	54.5±2.3^{b*}	62.1±2.8^{ab*}
ALH (μm)	3.0±6.1^{a}	2.7±0.1^{a}	2.7±0.2^{a}	1.1±0.5^{b}	1.1±0.5^{b}	2.7±0.1	2.9±0.1	2.9±0.0	2.5±0.1*	2.8±0.2*
BCF (Hz)	2.8±0.2^{a}	3±0.1^{a}	2.4±0.1^{a}	0.6±0.3^{b}	0.8±0.5^{b}	2.2±0.1^{b*}	2.6±0.1^{ab}	2.8±0.2^{a}	2.9±0.1^{a*}	2.8±0.1^{a*}
STR (%)	85.4±1.7^{a}	84.6±3.3^{a}	82.4±1.6^{a}	27.3±11.4^{b}	33.5±14^{b}	78.5±4.5^{b}	83±2.7^{ab}	86.8±1.6^{a}	86.2±1.8^{ab*}	86.9±2.4^{a*}
LIN (%)	61.2±3.9^{a}	62.9±5.4^{a}	53.7±6.6^{a}	9.1±4^{b}	13.9±7^{b}	55.6±4	57.3±4.5	63.4±2.3	65.4±2.4*	64.8±3.2*
RAP (%)	35±4.8^{a}	24.2±4.7^{b}	9.1±1.6^{c}	0.8±0.8^{c}	1.2±1.1^{c}	13.4±1.6^{b*}	20.8±4.8^{ab}	26.5±3.3^{a*}	19±4.7^{ab*}	28.5±3.4^{a*}
MED (%)	38.3±6.6^{a}	30.6±6.4^{ab}	18.5±4.1^{b}	2.0±1^{c}	2.9±1.5^{c}	27.4±6	29.1±5.1	27.4±5.8	32.5±7.2*	38.8±4.9*
SLOW (%)	5.2±0.4^{b}	11.3±1.3^{a}	11.4±0.9^{a}	3±1.2^{b}	7±3.8^{ab}	11.1±1.6^{ab*}	9.9±0.9^{ab}	8±0.9^{b*}	12.5±1.2^{a*}	10.2±1^{ab}
WOB (%)	71.4±3.7^{a}	75.2±3.6^{a}	64.6±7.2^{a}	18.4±6.5^{b}	21.8±8.7^{b}	72.4±3.6	68.5±3.7	72.9±1.5	76±2.5*	74.3±1.7*

a-d Superscripts indicates differences between concentrations ($P < 0.05$). * Indicates differences after the glucose supplementation ($P<0.05$). TM – Total Sperm Motility; PM – Progressive Motility; VAP – Average path velocity; VSL – Straight line velocity; VCL – Curvilinear velocity; ALH – Amplitude of lateral head displacement; BCF – Beat cross-frequency; STR – Straightness; LIN – Linearity; RAP - Percentage of rapid sperm, MED - Percentage of medium sperm; SLOW - Percentage of slow sperm; WOB - Wobble.

4.3.4 Experiment 4 – Effect of mitochondrial uncoupling and glycolysis stimulation on reactive oxygen species production

In the production of the reactive oxygen species, we highlight in Figure 9 the higher ROS generated by sperm treated with 3 µM of FCCP supplemented with glucose (332.9 ± 34.58µL) than with FCCP concentrations of 0.1 µM (213.2 ± 38.77µL), 1µM (191.44±50.39 µL) and 3µM (170.06 ± 49.34µL).

Figure 9 – Amount of O_2 generated by sperm treated with FCCCP in different concentrations (0µM, 0.1µM, 0.3µM, 1µM and 3µM) in absence or presence of glucose 5mM – São Paulo - 2016



[a-d] Superscripts indicates differences between concentrations (P < 0.05).

4.4 DISCUSSION

The aim of this study was to evaluate the role of mitochondria and the glycolytic pathway in the maintenance of ATP levels, the parameters of sperm movement and the production of reactive oxygen species in epididymal bovine sperm. To perform

this experiment, we submitted bovine sperm to mitochondrial uncoupling with FCCP to significantly reduce the synthesis of ATP by the mitochondria and evaluate the effect of this reduction in sperm functionality. Furthermore, we promoted stimulation of the glycolytic pathway by glucose addition concurrently with the mitochondrial uncoupling to assess whether glycolysis would be able to maintain the ATP levels, sperm kinetic patterns and oxidative homeostasis possibly harmed by mitochondrial depolarization.

The mitochondrial uncoupler FCCP is a lipophilic molecule with protonophore properties; in other words, it is capable of interacting with the inner mitochondrial membrane to allow pumped protons to return to the mitochondrial matrix, dissipating the proton gradient and influencing the mitochondrial chemiosmosis (TERADA, 1990; BAGKOS; KOUFOPOULOS; PIPERI, 2014). Indeed, in our experiment, we confirmed the depolarizing effect of the uncoupler FCCP in the dose-response curve (Experiment 1), where the minimum and maximum depolarization were obtained at concentrations of 0.3 and 100µM, respectively. From this experiment, we selected the lowest concentrations of the curve and an insufficient concentration to promote mitochondrial depolarization (FCCP 0.1, 0.3, 1 and 3 µM) to evaluate the effect of these treatments on mitochondrial ATP synthesis (Experiment 2). We used these concentrations because of the studies that demonstrate that mild mitochondrial depolarization is able to significantly reduce ATP synthesis (BAGKOS; KOUFOPOULOS; PIPERI, 2014). In addition, high concentrations of FCCP could cause mitochondrial disruptions, which would compromise cell homeostasis.

In experiment 2, we observed a significant reduction in ATP levels in groups treated with 0.3, 1 and 3µM of FCCP compared to the control group. ATP production in the mitochondria occurs by means of the coupling of two reactions: the transport of electrons throughout the respiratory chain and the proton gradient. This latest gradient is capable of storing energy, called proton motive force, which drives the synthesis of ATP through ADP and inorganic phosphate (LOWELL; SHULMAN, 2005). FCCP has a protonophore effect that will dissipate the proton gradient, thereby reducing ATP synthesis, as noted in our results. On the other hand, the groups that were treated with these same FCCP concentrations but were supplemented with glucose had higher levels of ATP, similar to the control group. From these results, we can suggest that the glycolytic pathway, after being stimulated, is able to maintain ATP levels in bovine epididymal sperm. In fact, our

results were consistent with a previous study in boars, which demonstrated that sperm mitochondria accounts for only 5% of energy production, while the glycolytic pathway contributes to 95% (MARIN et al., 2003). Additionally, species such as mice may use ATP from glycolysis and mitochondrial respiration depending on their biological conditions without changing sperm functionality or sperm ATP levels (PASUPULETI, 2007).

In experiment 3, we observed a very similar pattern of results to experiment 2. The motility and spermatic movement patterns were affected by mitochondrial uncoupling. However, stimulation of the glycolytic pathway maintained sperm kinetic patterns, even with cells undergoing mitochondrial uncoupling. These results suggest that for bovine sperm, there is a close relationship between motility and ATP levels. However, this relationship is still a matter of controversy. In accordance with our study, Mukai and Okuno (2004) verified that ATP levels and flagellar beating remained constant when mouse sperm mitochondria were uncoupled concurrently with the supplementation of substrates for glycolysis. Additionally, Krzyzosiak, Molan and Vishwanath (1999) also observed that bovine sperm are capable of maintaining similar motility patterns in both aerobic and anaerobic conditions, assuming that glycolysis is capable of maintaining sperm motility. On the other hand, Ramió-Lluch et al. (2014) demonstrated that the inhibition of ATP synthase impairs sperm motility, while intracellular ATP levels remain unchanged. Therefore, the author suggested an unknown essential mitochondrial mechanism responsible for motility maintenance that does not rely only on the maintenance of ATP levels. The variations in the results of the different experiments seem to be related to the species involved and the biological conditions to which such cells have been subjected (STOREY, 2008; AMARAL et al., 2013). Therefore, there is a need for further studies to elucidate these mechanisms.

Regarding experiment 4, we observed that the groups treated with FCCP at 1 and 3μM in the absence of glucose had a lower production of reactive oxygen species (ROS) than the groups treated with the same concentrations in the presence of glucose. The reactive oxygen species produced by sperm play a key role in many physiological processes such as hyperactivation (DE LAMIRANDE; CAGNON, 1993), capacitation (AITKEN et al., 2004) and the interaction between the sperm and oocyte (AITKEN et al., 1995). The fact that the groups treated with FCCP and glucose did not differ from the control group suggests that glycolysis stimulation is able to

maintain the physiological ROS production and, ultimately, oxidative balance. Moreover, the ability of FCCP in the absence of glucose to reduce ROS production reveals a possible therapeutic potential for preventing the release of excessive reactive oxygen species. This ability to prevent ROS production may be due to the increase of the electron transport rates accompanied by a reduction in mitochondrial intermediate states able to donate electrons to oxygen (CUNHA et al., 2011). Furthermore, studies have demonstrated that the reduction in ATP synthesis by mitochondria is accompanied by a reduction in ROS production (NEWSHOLME et al., 2007). In fact, studies have shown this ability of mitochondrial uncouplers in somatic cells (VINCENT et al., 2004; MAILLOUX; HARPER, 2011). However, this therapeutic effect should be further studied in spermatozoa.

In conclusion, the glycolytic pathway after stimulation is capable of maintaining ATP levels, sperm kinetic patterns and oxidative balance of bovine epididymal spermatozoa submitted to mitochondrial uncoupling.

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Chapter 3

5 CHAPTER 3 – Mitochondrial uncoupling during sperm cryopreservation in rams: Effect on sperm functionality, bioenergetics and oxidative homeostasis

ABSTRACT

Sperm cryopreservation is a key process in reproductive biotechnologies. However, it is known that this process causes damages to the spermatozoa, and thus reducing sperm quality after thawing. Due to mitochondria being the main source of reactive oxygen species (ROS), we believe that mitochondrial dysfunctions during sperm cryopreservation is the cause of excessive release of pro-oxidative agents causing sperm injury after thawing. Therefore, we hypothesized that by mitochondrial uncoupling during sperm cryopreservation and stimulating the glycolytic pathway to supply ATP levels, possibly reduced by mitochondrial uncoupling, we can prevent the excessive ROS release during this procedure, improving the post-thawing sperm quality. In accordance with our hypothesis, we observed a higher percentage of motile cells, higher mitochondrial membrane potential, lower lipid peroxidation and lower DNA susceptibility to acid denaturation in spermatozoa submitted to mitochondrial decoupling concurrently with the glycolysis stimulation.

5.1 INTRODUCTION

Sperm cryopreservation is considered a key process in reproductive biotechnologies (HAMMERSTEDT; GRAHAM; NOLAN, 1990; ZAPZALKA; REDMON; PRYOR, 1999; HOLT, 2000). However, it is known that this technique results in a decrease in sperm quality. A potential cause of this decreased sperm quality is the oxidative stress (O.E.) during the cryopreservation process. Because mitochondria are the main source of pro-oxidative release factors, this organelle has been suggested to play a central role in oxidative imbalance (AGARWAL et al., 2014). Therefore, mitochondrial dysfunction during cryopreservation is possibly the

origin of the excessive production of reactive oxygen species (ROS), consequently causing post-thaw sperm damage (O'CONNELL; MCCLURE; LEWIS, 2002; SARIOZKAN et al., 2009; THOMSON et al., 2009). In addition, a reduction in the antioxidant capacity of sperm after cryopreservation has been verified, which predisposes these cells to oxidative stress (BILODEAU et al., 2000).

Thus, several studies have been using antioxidant therapy in sperm samples submitted to cryopreservation, aiming for the prevention of O.E. caused by mitochondrial injury (ASKARI et al., 1994; BILODEAU et al., 2001; FERNÁNDEZ-SANTOS et al., 2007; TAYLOR et al., 2009). However, it is necessary to find the optimal concentration of these antioxidants to maintain oxidative balance since the reactive oxygen species play a physiological role in the spermatozoa. In addition, each antioxidant acts on the elimination of a specific ROS. Therefore, for these treatments to be effective, the ideal concentrations would need to be associated with the performance of the antioxidants, which may make this therapy infeasible.

However, some specific mitochondrial protectors during cryopreservation for improving post-thaw sperm quality have been suggested (SCHOBER et al., 2007). A possible alternative would be a mild mitochondrial depolarization induced by uncouplers of oxidative phosphorylation in the cryopreservation process to ensure that in any mitochondrial dysfunction, there is a reduction in the release of pro-oxidative agents to improve sperm quality. In fact, the activities of some uncouplers were identified in physiological processes of somatic cells and were shown to even cause a reduction in oxidative stress (VINCENT et al., 2004; BRAND; ESTEVES, 2005). However, scientific studies similar to these with more details on spermatozoa are scarce in the literature, especially in regards to sperm ruminants, which are widely cryopreserved and extremely important for global livestock production.

Therefore, the aim of our study is to promote mitochondrial uncoupling of ram sperm during the cryopreservation process and verify the effect of this procedure on sperm functionality, bioenergetics and oxidative homeostasis, aiming to improve post-thaw sperm quality.

5.2 MATERIAL AND METHODS

The experiment was conducted using animals of the Department of Animal Reproduction of the College of Veterinary Medicine and Animal Science from the University of Sao Paulo. Experiments were conducted according to the guidelines of ethics in animal experiments and approved by the ethics committee of this institution (protocol number 7978040914). Unless otherwise stated, all chemicals utilized in this study were purchased from Sigma Chemical® (St. Louis, MO, USA).

5.2.1 Experimental design

To promote mitochondrial uncoupling, we used the uncoupler carbonyl cyanide meta-chlorophenyl hydrazine (CCCP). Despite the possible beneficial effects of CCCP, this compound may cause mitochondrial alterations such as a reduction in ATP synthesis and, consequently, changes in bioenergetics and sperm function. Thus, we simultaneously stimulated the glycolytic pathway along with the mitochondrial uncoupling in order to cause a possible reduction in ATP synthesis by oxidative phosphorylation.

For this study, ejaculates were collected from eight (N=8) healthy and sexually mature rams by means of an artificial vagina. A minimal motility of 70% was considered to be an inclusion factor. Immediately after collection, sperm samples were diluted in cryopreservation medium (BotuBov® Botupharma, Brazil) to a final concentration of 100 x 106 spermatozoa / mL. The diluted semen was then divided into 8 aliquots: one untreated aliquot considered to be the control group (CCCP 0µM) and a second aliquot treated only with glucose (glucose 5mM); the remains were treated with three concentrations of the mitochondrial uncoupler CCCP in the presence or absence of glucose (CCCP 1µM, CCCP 10µM, CCCP 20µM, CCCP 1µM + glucose 5mM, CCCP 10µM + glucose 5mM and CCCP 20µM + glucose 5mM). Subsequently, the groups were submitted to sperm cryopreservation and thawing.

5.2.2 Sperm cryopreservation

The diluted sperm samples were packaged in 0.5 mL straws, and then, the samples originally kept at 37°C were submitted to a slow cooling to 5°C for 2 hours. After this period, the semen samples were kept in nitrogen vapor (-70°C) for 20 min and sequentially immersed and stored in liquid nitrogen. After at least a week, the samples were then thawed at 37°C for 30 seconds in a water bath and submitted to the subsequent analysis.

5.2.3 Sperm analysis

After sperm thawing, the samples were subjected to computer analysis of sperm kinetic patterns, functional tests (integrity of plasmatic and acrosomal membranes, mitochondrial activity, mitochondrial membrane potential and DNA integrity), oxidative status evaluation (reactive oxygen species detection and evaluation of susceptibility to lipid peroxidation)

5.2.3.1 Computer analysis of sperm kinetics patterns

Sperm kinetic patterns were assessed using the Computer Assisted Sperm Analysis (CASA; Hamilton-Thorne®, Ivos 12.3, USA). The following variables were considered: motility (%), progressive motility (%), VAP (average path velocity, µm/s), VSL (straight-line velocity, µm/s), VCL (curvilinear velocity, µm/s) ALH (amplitude of lateral head displacement, µm), BCF (beat cross-frequency, Hz) STR (straightness, %) and LIN (linearity, %). In addition to these parameters, the sperm velocity was also divided into four groups: rapid (VAP> 50µm /s, %), medium (30 µm / s <VAP <50µm / s; %), slow (VAP <30µm /s or VSL <15µm / s, %) and static (%) (GOOVAERTS et al., 2006).

5.2.3.2 *Sperm functional tests*

The sperm functional tests were performed according to the methodology of Castro et al. (2015) using flow cytometry (Guava EasyCyteTM Mini System, Guava® Technologies, 190 Hayward, CA, E.U.A.), with the exception of the use of the cytochemical assay 3'3 diaminobenzidine (DAB assay). This equipment contains a blue laser, which operates at 488 nm and emits a 20-mW visible laser radiation. A total of 10,000 events per sample were analysed, and data corresponding to yellow (PM1 photodetector – 583 nm), red (PM2 photodetector – 680 nm) and green fluorescent signals (PM3 photodetector – 525 nm) were recorded after a logarithmic amplification. All data were analysed by FlowJo® v10.2 software, except for DNA integrity, which was evaluated using FlowJo®v8.7 software.

Plasmatic and acrosomal membranes were assessed by propidium iodide (PI) and FITC conjugated with Pisum sativum agglutinin (FITC-PSA) probes, respectively. This association of fluorophores divided sperm populations in four groups: intact membrane and intact acrosome (IMIA), intact membrane and damaged acrosome (IMDA), damaged membrane and intact acrosome (DMIA) and damaged membrane and damaged acrosome (DMDA). The procedure was performed with 185,000 cells diluted in modified TALP and stained with 0.5 mg/ml PI in NaCl 0.9% and 100 mg/ml FITC-PSA (FITC-PSA L-0770) in a sodium azide solution at 10% in DPBS. Samples were analysed by flow cytometry after 10 min, excited at 488 nm and detected at 630–650 nm (PI) and 515–530 nm (FITC).

Mitochondrial membrane potential (MMP) was assessed using the JC-1 probe (5,5',6,6'-tetrachloro-1,1',3,3' 201 -tetraethyl- benzimidazolylcarbocyanine chloride; Invitrogen, Eugene, OR, USA). To perform the technique, 187,500 spermatozoa diluted in 12.5µl TALP medium were added to 0.5µl of the fluorescent probe JC-1 (76.5mM) and incubated at 37°C for 5 min. Samples were classified into sperm with high (JC-1 high), intermediate (JC-1 intermediate) and low (JC-1 low) mitochondrial membrane potential.

We evaluated mitochondrial activity by means of the cytochemical technique using the reagent 3'3 diaminobenzidine (DAB assay) according to the methodology used for Losano et al. (2015). In this technique, 3'3 diaminobenzidine is oxidized by the cytochrome c enzyme and forms a brown coloured complex that is deposited on

active mitochondria (HRUDKA, 1987). Briefly, 20µL of semen was incubated with 20µL of 3'3 diaminobenzidine in amber microcentrifuge tubes for 1 hour in a water bath at 37°C. After incubation, the mixture was smeared on microscopy slides in the dark. Slides were subsequently fixed in 10% formaldehyde for 10 min. Analysis was performed using phase-contrast microscopy at 1000 x magnification under immersion oil. One hundred cells were counted and classified into 4 classes according to the percentage of stained midpiece mitochondria: completely stained, indicating high mitochondrial activity (DAB I); most of the midpiece stained, indicating medium activity (DAB II); most of the midpiece unstained, indicating low activity (DAB III); and midpiece completely unstained, indicating absence of mitochondrial activity (DAB IV).

The chromatin stability assay was performed using the sperm chromatin structure assay (SCSA) described by Evenson and Jost (2000). To perform this technique, 375,000 cells were incubated with TNE buffer (Tris-HCl 0.01 M, NaCl 0.15 M, EDTA 1 mM and distilled water, pH 7.4) and acid detergent (HCl 0.08 M, NaCl 0.15 M, Triton X-100 0.1% in distilled water, pH 1.2). After 30 seconds, acridine orange (stock solution 6µg/mL) was added, and each sample was analysed after 5 min of incubation at 37°C, excited at 488 nm and detected at 630-650 nm (red) and 515-530 nm (green).

5.2.3.3 Oxidative status evaluation

To perform the reactive oxygen species detection, we used 2', 7'-dichlorfluorescein-diacetate (DCFH) and CellROX green fluorophores according to the methodology used by (CASTRO et al., 2016). Additionally, we evaluated the susceptibility to lipid peroxidation utilizing the TBARS assay.

CellROX® green (Molecular Probes, Eugene, OR, E.U.A.) is a fluorescent probe that penetrates the cell and, when oxidized by intracellular free radicals, binds to DNA, emitting a more intense green fluorescence. To perform this technique, 187,500 cells were stained with CellROX® green (final concentration of 5µM) for 30 min at 37°C, and 10 min prior to the end of this incubation, PI was added to a final concentration of 6µM. Samples were analysed by flow cytometry, excited at 488 nm and detected at 630-650 nm for PI and 515-530 nm for CellROX® green. For data

analysis, we selected the population of cells that were without membrane alteration and were stressed (PI- and CellROX+).

To perform ROS detection by DCFH fluorescent probe, 187,500 cells were added to a solution containing DCFH (9.3 μ M) and propidium iodide (PI, 6 μM) in the dark at 37°C. Samples were analysed by flow cytometry after 5 min, excited at 488 nm, and detected at 630– 650 nm (PI) and 515–530 nm (DCFH). For data analysis, we selected the population of cells that were without membrane alteration and were stressed (PI- and DCFH+).

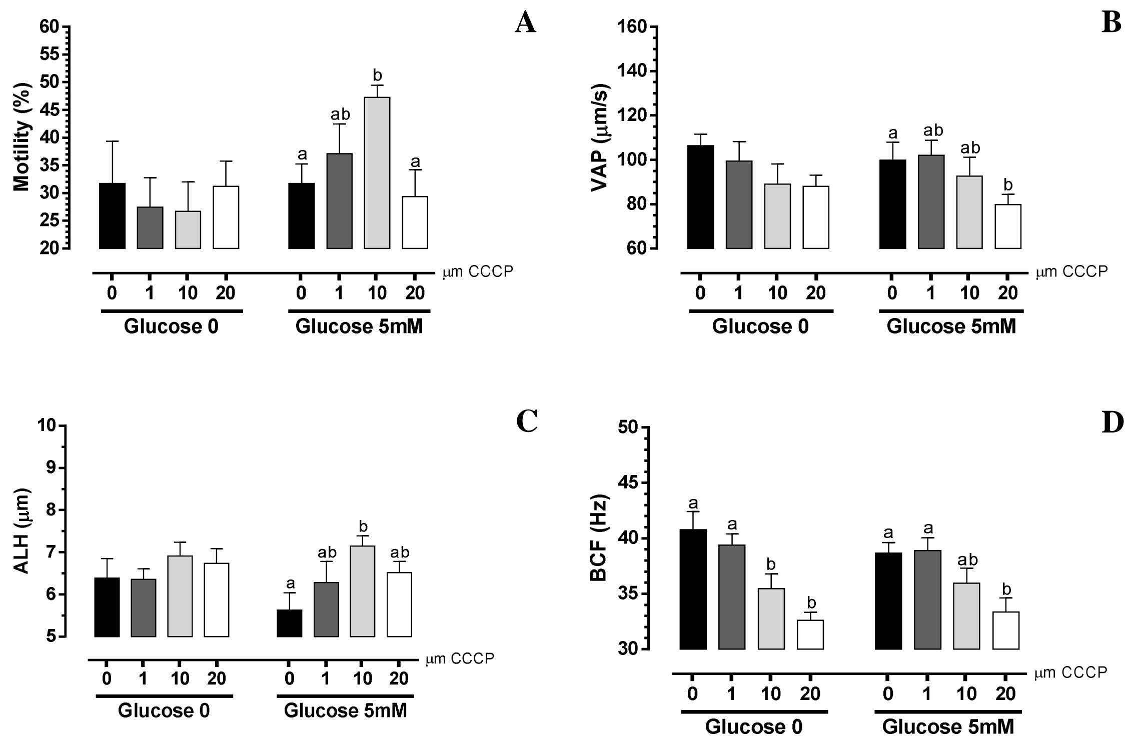
The TBARS assay (Thiobarbituric Acid Reactive Substances) was conducted according to the methodology adapted by Nichi et al. (2007). This technique was performed after the induction of lipid peroxidation by the incubation of 200µl of semen with 50 µL of ascorbic acid (20mM) and 50 µL of iron sulfate (4mM) in a water bath at 37°C for 90 min. After induction, trichloroacetic acid 10% (600 µL) was added. Samples were then centrifuged at 20800 x g for 15 min (5° C) for precipitation of proteins and debris. Subsequently, 800µL of the supernatant were recovered and transferred to cryotubes. Thiobarbituric acid 1% (TBA; 800 µL) was added to the tubes, which were then incubated at 95°C in a water bath for 15 min. In this reaction, malondialdehyde (MDA; primary product of lipid peroxidation) and TBA react, producing a complex pinkish in colour. The amount of colour was quantified using a spectrophotometer (Ultrospec 3300 Pro® Amersham Biosciences, USA) at a wavelength of 532 nanometers. The susceptibility to lipid peroxidation was expressed in nanograms of TBARS / 10^6 spermatozoa.

5.3 RESULTS

We can observe the results of all variables in the Tables 3 and 4 available in the supplementary material. With regards to the sperm kinetics, we observed a significantly higher percentage of motile cells in the samples treated with CCCP 10µM + glucose 5mM than in the samples treated only with glucose (glucose 5mM) and those treated with CCCP 20µM + glucose 5mM (Figure 10A). The highest concentration of CCCP with glucose (CCCP 20µM + glucose 5mM) in turn reduced the average path velocity (VAP) compared to the samples treated only with glucose

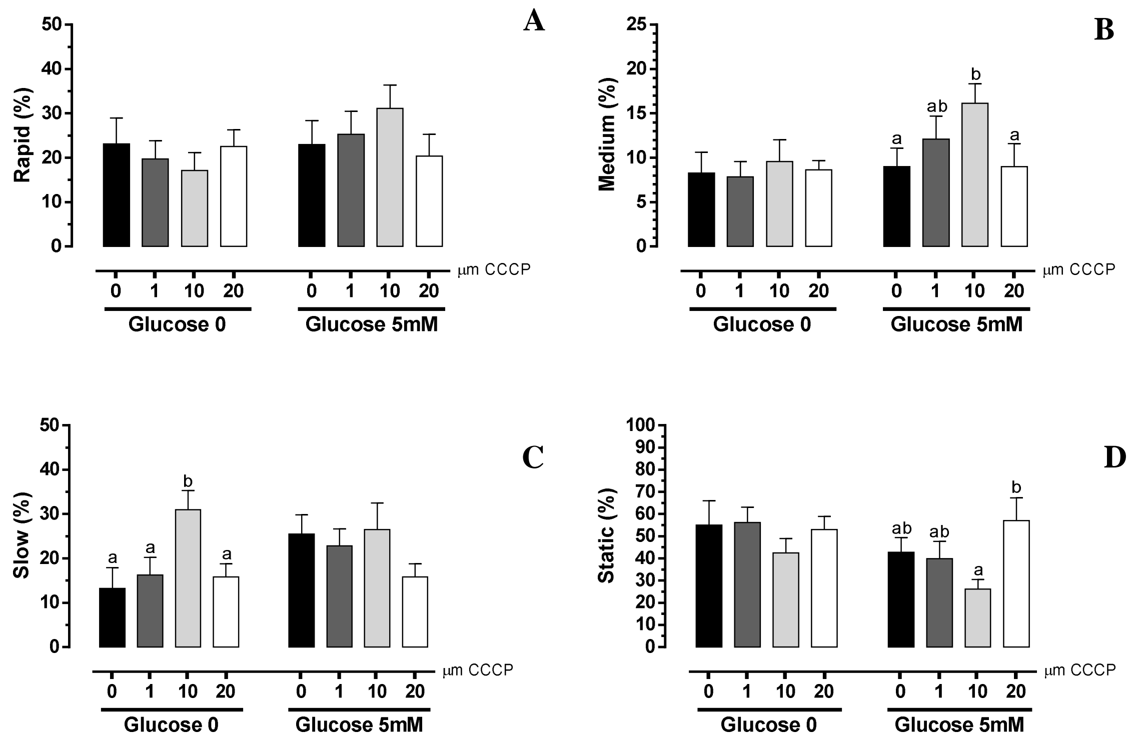
(glucose 5mM, Figure 10B). We observed a higher amplitude of lateral head displacement (ALH) in the samples treated with 10μM CCCP + glucose 5mM than those treated with glucose 5mM alone (Figure 10C). Additionally, the concentrations of 10 and 20 μM of CCCP without the addition of glucose promoted a reduction in cross-beat frequency (BCF, Figure 1 D). Moreover, the CCCP 20μM + glucose 5mM group showed a lower beat cross-frequency (BCF) than the samples treated with CCCP 1μM + glucose 5mM and the glucose 5mM group (Figure 10D).

Figure 10 - Effect of mitochondrial uncoupling (CCCP) and glycolysis stimulation (glucose) on spermatic kinetics variables: motility (A), VAP (B), ALH (C) and BCF (D) – São Paulo - 2016



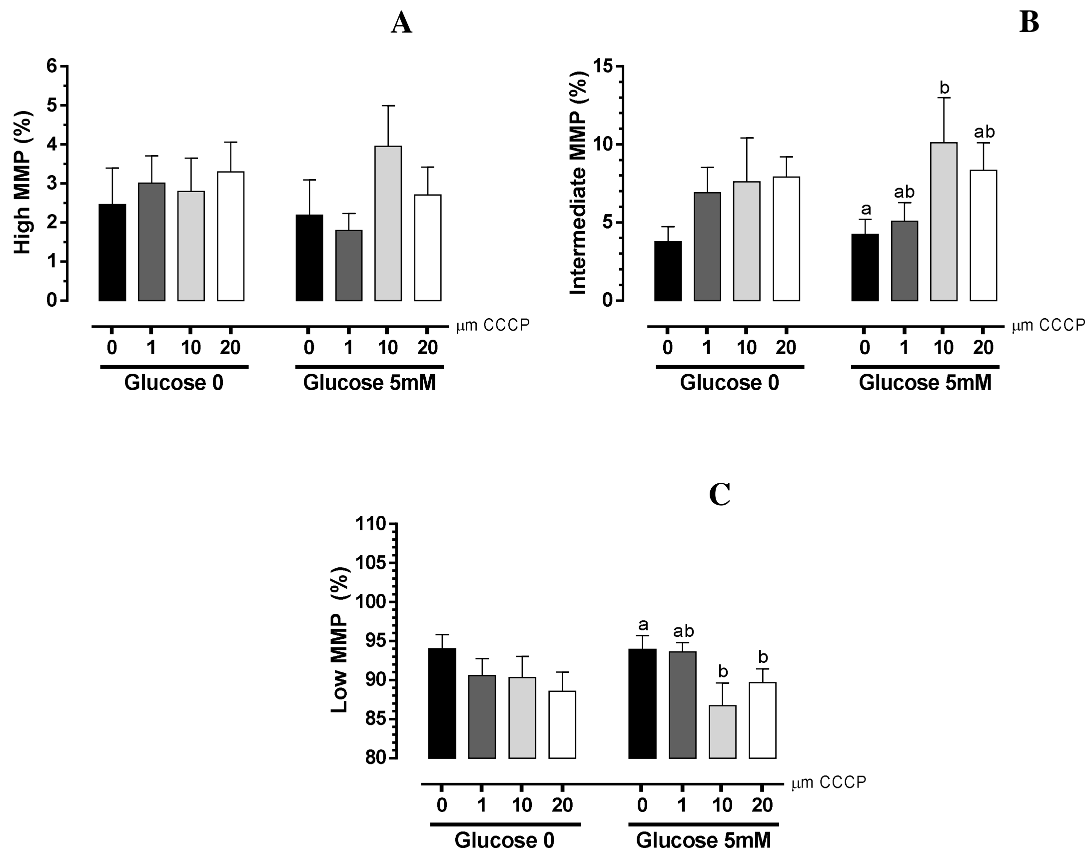
In respect to the sperm velocities, the group treated with 10μM CCCP + glucose 5mM promoted a higher prevalence of cells with medium velocity than samples treated only with glucose and the samples treated with 20μM CCCP + glucose 5mM (Figure 11B). Additionally, we observed a higher percentage of cells with slow velocity in the samples treated with 10μM of CCCP (Figure 11C). On the other hand, the samples treated with 10μM CCCP with the addition of glucose showed a lower percentage of static cells than the group treated with 20μM CCCP + glucose 5mM (Figure 11D).

Figure 11 – Effect of mitochondrial uncoupling (CCCP) and glycolysis stimulation (glucose) on sperm velocities: rapid (A), medium (B), slow (C) and static (D) – São Paulo - 2016



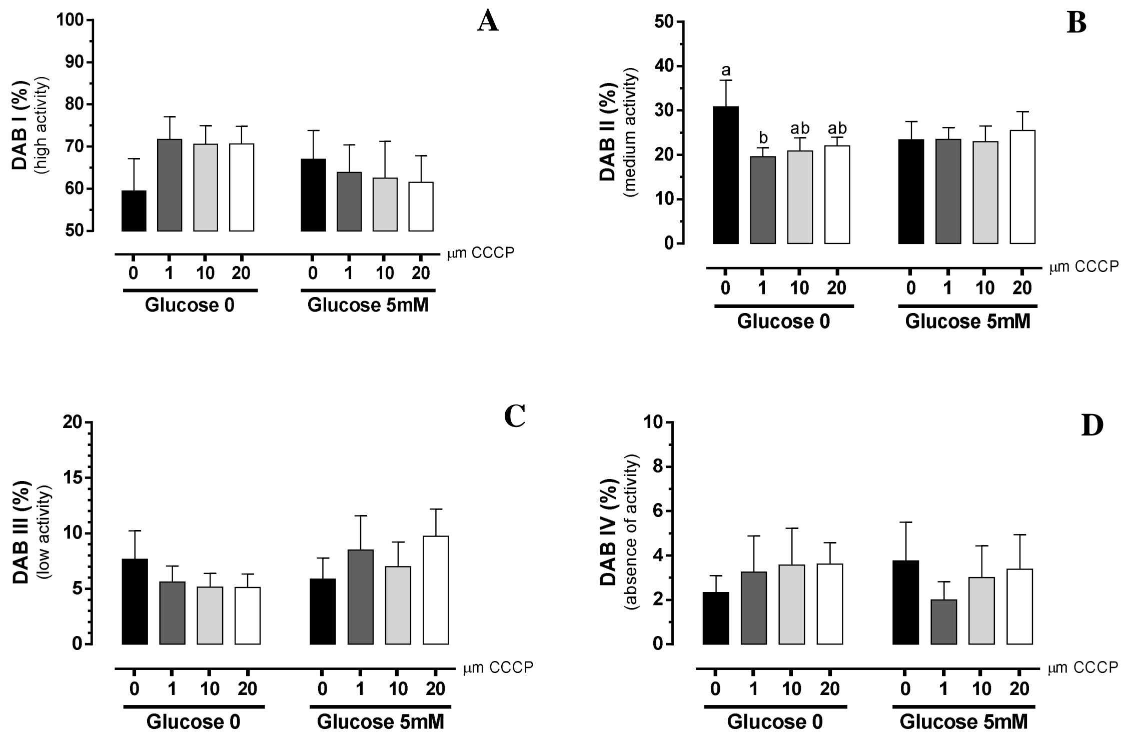
We observed an increase in the percentage of cells with intermediate mitochondrial membrane potential (MMP, Figure 12B) and a decrease in the percentage of cells with low MMP (Figure 12C) in the samples treated with 10μM CCCP + glucose 5mM.

Figure 12 - Effect of mitochondrial uncoupling (CCCP) and glycolysis stimulation (glucose) on mitochondrial membrane potential: High (A), intermediate (B) and low mitochondrial membrane potential (C) – São Paulo - 2016



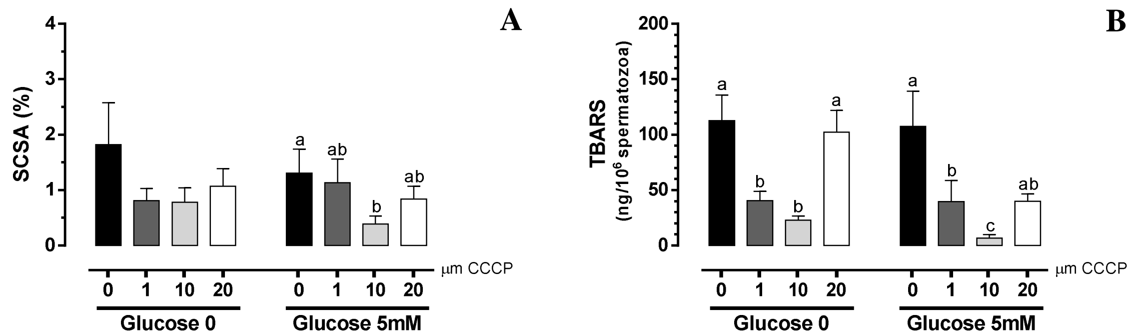
Lastly, the treatment with the lowest concentration of CCCP (1μM) in the absence of glucose resulted in a reduction in the percentage of cells with intermediate mitochondrial activity compared to the samples not treated with CCCP (Figure 13B).

Figure 13 - Effect of mitochondrial uncoupling (CCCP) and glycolysis stimulation (glucose) on mitochondrial activity: high (DABI A), intermediate (DABII, B), low (DABIII, C) and absence of mitochondrial activity (DABIV, D) – São Paulo - 2016



Furthermore, we observed a significant reduction in the susceptibility to lipid peroxidation of the samples treated with 1and 10µM of CCCP (Figure 14A). In addition, the treatment with 10µm CCCP + glucose 5mM reduced the susceptibility to lipid peroxidation compared to the samples treated with glucose 5mM (Figure 14A). Lastly, the samples treated with 10µm CCCP + glucose 5mM showed lower DNA susceptibility to acid denaturation (Figure 14B).

Figure 14 - Effect of mitochondrial uncoupling (CCCP) and glycolysis stimulation (glucose) on DNA susceptibility to acid denaturation (SCSA, A) and susceptibility to lipid peroxidation (TBARS,B) – São Paulo - 2016



5.4 DISCUSSION

Although sperm cryopreservation is a key process for the implementation of several reproductive biotechnologies, it is known that this procedure reduces the percentage of viable cells post thawing (HAMMERSTEDT; GRAHAM; NOLAN, 1990; ZAPZALKA; REDMON; PRYOR, 1999; HOLT, 2000). A possible cause for this effect may be the oxidative stress promoted by cryopreservation. Because the mitochondrion is the main source for the release of ROS, dysfunction in this organelle during processing and seminal cryopreservation is involved in oxidative imbalance and the consequent reduction in sperm quality (O'CONNELL; MCCLURE; LEWIS, 2002; SARIOZKAN et al., 2009; THOMSON et al., 2009). A likely alternative would be to use a molecule that could act in the mitochondria during sperm cryopreservation to prevent the excessive release of reactive oxygen species. Studies in somatic cells demonstrate that a mild mitochondrial uncoupling can prevent oxidative damage (VINCENT et al., 2004; BRAND; ESTEVES, 2005). Therefore, we hypothesized that mitochondrial uncoupling in ram sperm during cryopreservation can prevent excessive ROS release and consequently improve post-thaw sperm quality. To confirm our hypothesis, we submitted the sperm samples to increasing concentrations of the mitochondrial uncoupler CCCP in the presence or absence of glucose. We used glucose to stimulate the glycolytic pathway concurrently with the mitochondrial uncoupling. This procedure was performed to

investigate the possible compensation in the supply of energy by glycolysis, as this mitochondrial approach may reduce ATP synthesis for this organelle. In fact, in our study we observed an improvement in several sperm attributes when we used this therapeutic strategy for sperm cryopreservation in rams.

Mitochondrial disruption is related to several diseases such as neurodegenerative diseases (LIN; BEAL, 2006), diabetes (LOWELL; SHULMAN, 2005), cancer (MODICA-NAPOLITANO; SINGH, 2004) and infertility (TROIANO et al., 1998; GALLON et al., 2006). Furthermore, studies demonstrate that a mild mitochondrial uncoupling prevents dysfunction in this organelle (VINCENT et al., 2004; BRAND; ESTEVES, 2005). This occurs because, due to the mitochondrial uncoupling, there is an increase in the electron transport rates and a decrease in oxygen tension, preventing excessive pro-oxidant release (CUNHA et al., 2011). We believe that sperm mitochondrion with a high mitochondrial membrane potential (MMP) have a greater potential to produce and release reactive oxygen species (ROS) during sperm cryopreservation, which can lead to oxidative imbalance. On the other hand, a slight reduction in MMP could protect the sperm mitochondria during cryopreservation and reduce excessive ROS release. In our study, treatment with 10μM CCCP associated with 5mM glucose promoted a protective effect in the mitochondria during cryopreservation, resulting in a higher percentage of cells with a medium mitochondrial membrane potential. Furthermore, the percentage of cells with high and low mitochondrial membrane potentials did not differ between the experimental treatments.

Studies carried out in different species showed a negative correlation between both oxidative stress and high mitochondrial activity as well as between the occurrence of this stress and the sperm DNA integrity, indicating that these variables are interrelated, forming a single pathogenic mechanism (BLUMER et al., 2008; BLUMER et al., 2012; SIMÕES et al., 2013; AGARWAL et al., 2014). In fact, studies show that oxidative stress, is the main cause of sperm DNA fragmentation post-spermatogenesis (SAKKAS; ALVAREZ, 2010). In addition, the lipid peroxidation products (i.e., aldehydes) may be as deleterious to sperm DNA as ROS (PERIS et al., 2007; AITKEN et al., 2013). Thus, we can strengthen this theory with our results, as the same group that conferred a mitochondrial protective effect (CCCP 10μM + 5mM glucose) promoted the protection of DNA and reduced sperm susceptibility to lipid peroxidation (i.e., consequence of oxidative stress).

The plasma membrane of spermatozoa is rich in polyunsaturated fatty acids (PUFAs) (PARKS; HAMMERSTEDT, 1985). PUFAs are essential for maintaining the fluidity of the sperm membrane, which is extremely important for the physiological processes of the sperm such as motility and interaction with the oocyte (PARKS; HAMMERSTEDT, 1985; AGARWAL; SALEH; BEDAIWY, 2003). Moreover, these PUFAs are easily oxidized, which makes the spermatozoa susceptible to oxidative stress (VERNET; AITKEN; DREVET, 2004; NICHI et al., 2007). Thus, if lipid peroxidation is prevented, sperm kinetics may be preserved. Furthermore, polyunsaturated fatty acids may play a protective role in sperm cryopreservation (WATERHOUSE et al., 2006). Curiously, we observed a higher motility in the group treated with 10µM CCCP in the presence of 5mM glucose than in the untreated group. This could be due to protection of this treatment against lipid peroxidation during cryopreservation. Moreover, treatments with CCCP in the absence of glucose did not appear to be effective for improving sperm kinetics. In these groups, we observed lower BCF in the samples treated with 10 or 20µM CCCP and a greater percentage of cells with low velocity in the samples treated with 10µM CCCP. For some species, the glycolytic pathway appears to be more important for sperm kinetics than oxidative phosphorylation. In fact, Mukai and Okuno (2004) demonstrated that murine spermatozoa submitted to mitochondrial uncoupling and stimulated with glucose were capable of maintaining the flagellar beating. On the other hand, with stimulation of the oxidative phosphorylation and inhibition of the glycolytic pathway, the flagellar beating reduced quickly. Recently, a work performed by our group showed that the glycolytic pathway, once stimulated, is able to maintain motility and ATP levels of bovine spermatozoa as they undergo mitochondrial uncoupling (unpublished data). Therefore, the glycolytic pathway seems to replace the physiological role of mitochondria in the maintenance of sperm kinetics, which favours therapy against cryoinjuries by mitochondrial uncoupling.

Despite the need for more detailed studies using mitochondrial uncouplers during sperm cryopreservation to prove the efficacy of these treatments in the fertilization process, we suggest the use of this therapy to improve motility and post-thaw sperm attributes due to mitochondrial protection. Furthermore, this treatment appears to only be effective upon stimulation of the glycolytic pathway. Perhaps this effect was observed due to the glycolysis offset the energy demands required for homeostasis and ram sperm functionality.

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APENDEX A: Supplementary material

Table 3 - Effect of mitochondrial uncoupling without glycolysis stimulation during sperm cryopreservation on spermatozoa variables – São Paulo - 2016

	CCCP 0µM	CCCP 1µM	CCCP 10µM	CCCP 20µM
VAP (µm/s)	106.36 ± 5.23	99.50 ± 8.83	89.06 ± 9.08	87.99 ± 5.14
VCL (µm/s)	95.49 ± 6.29	87.41 ± 9.55	73.93 ± 8.72	75.86 ± 4.38
VSL (µm/s)	172.83 ± 4.39	164.09 ± 9.45	156.74 ± 13.08	152.63 ± 11.01
ALH (µm)	6.39 ± 0.46	6.36 ± 0.25	6.91 ± 0.33	6.74 ± 0.35
BCF (Hz)	40.77 ± 1.65^{a}	39.39 ± 1.01^{a}	35.46 ± 1.34^{b}	32.63 ± 0.73^{b}
STR (%)	86.57 ± 2.99	82.25 ± 3.34	78.14 ± 2.52	84.25 ± 1.16
LIN (%)	54.57 ± 3.44	52.38 ± 3.39	46.43 ± 2.25	50.38 ± 1.12
MOTILE (%)	31.71 ± 7.69	27.50 ± 5.27	26.71 ± 5.33	31.25 ± 4.55
PROGRESSIVE (%)	19.86 ± 5.08	16.75 ± 3.49	13.57 ± 3.54	19.50 ± 3.56
RAPID (%)	23.14 ± 5.85	19.75 ± 4.09	17.14 ± 4.03	22.50 ± 3.83
MEDIUM (%)	8.29 ± 2.36	7.88 ± 1.74	9.57 ± 2.50	8.63 ± 1.07
SLOW (%)	13.29 ± 4.68^{a}	16.25 ± 4.02^{a}	31.00 ± 4.36^{b}	15.88 ± 2.89^{a}
STATIC (%)	55.00 ± 11.08	56.13 ± 7.12	42.43 ± 6.58	53.13 ± 5.84
DAB I (%)	59.50 ± 7.67	71.75 ± 5.33	70.57 ± 4.39	70.63 ± 4.19
DAB II (%)	30.83 ± 5.69^{a}	19.63 ± 2.75^{b}	20.86 ± 3.16^{ab}	22.00 ± 2.07^{ab}
DAB III (%)	7.67 ± 2.58	5.63 ± 1.45	5.14 ± 1.26	5.13 ± 1.20
DAB IV (%)	2.33 ± 0.76	3.25 ± 1.63	3.57 ± 1.67	3.63 ± 0.96
HIGH MMP (%)	2.46 ± 0.93	3.01 ± 0.70	2.80 ± 0.85	3.30 ± 0.76
INTER MMP (%)	3.77 ± 0.96	6.91 ± 1.62	7.60 ± 2.82	7.93 ± 1.27
LOW MMP (%)	94.04 ± 1.81	90.59 ± 2.15	90.31 ± 2.74	88.56 ± 2.48
TBARS (ng/10^{6} sptz)	112.72 ± 23.09^{a}	40.57 ± 8.22^{b}	22.78 ± 3.88^{b}	102.39 ± 19.47^{a}
CELLROX (%)	42.93 ± 3.07	36.71 ± 1.63	43.20 ± 6.77	42.16 ± 3.76
DCFH (%)	7.45 ± 2.31	18.03 ± 9.09	14.47 ± 5.01	6.78 ± 1.61
SCSA (%)	1.82 ± 0.76	0.81 ± 0.22	0.78 ± 0.26	1.07 ± 0.32
DMDA (%)	44.53 ± 8.22	46.50 ± 7.24	44.37 ± 7.33	43.19 ± 7.50
IMDA (%)	0.96 ± 0.10	1.08 ± 0.22	1.19 ± 0.24	1.40 ± 0.32
DMIA (%)	42.63 ± 6.23	41.04 ± 5.08	39.40 ± 3.87	42.35 ± 5.50
IMIA (%)	11.84 ± 2.41	11.40 ± 2.84	15.04 ± 5.15	13.04 ± 2.75

Table 4 - Effect of mitochondrial uncoupling and glycolysis stimulation during sperm cryopreservation on spermatozoa variables – São Paulo - 2016

	CCCP 0μM	CCCP 1μM	CCCP 10μM	CCCP 20μM
VAP (μm/s)	99.94 ± 7.96[a]	102.01 ± 6.85[ab]	92.70 ± 8.53[ab]	79.74 ± 4.64[b]
VCL (μm/s)	89.31 ± 8.72	88.79 ± 8.83	78.51 ± 7.69	67.90 ± 5.86
VSL (μm/s)	154.19 ± 8.01	161.74 ± 6.96	159.43 ± 14.07	138.85 ± 6.51
ALH (μm)	5.64 ± 0.41[a]	6.29 ± 0.51[ab]	7.16 ± 0.24[b]	6.53 ± 0.26[ab]
BCF (Hz)	38.68 ± 0.95[a]	38.93 ± 1.15[a]	35.97 ± 1.35[ab]	33.38 ± 1.25[b]
STR (%)	84.00 ± 3.23	82.75 ± 3.34	82.86 ± 2.25	81.63 ± 3.58
LIN (%)	57.38 ± 5.02	50.29 ± 3.11	51.29 ± 2.49	48.38 ± 3.46
MOTILE (%)	31.75 ± 3.54[a]	37.13 ± 5.35[ab]	47.29 ± 3.20[b]	29.38 ± 4.86[a]
PROGRESSIVE (%)	20.25 ± 4.90[a]	21.38 ± 5.01[ab]	24.14 ± 3.89[b]	18.00 ± 4.19[a]
RAPID (%)	23.00 ± 5.42	25.25 ± 5.23	31.14 ± 5.22	20.38 ± 4.94
MEDIUM (%)	9.00 ± 2.10[a]	12.13 ± 2.58[ab]	16.14 ± 2.21[b]	9.00 ± 2.61[a]
SLOW (%)	25.50 ± 4.35	22.88 ± 3.81	26.57 ± 5.95	13.63 ± 4.11
STATIC (%)	42.75 ± 6.66[ab]	39.88 ± 7.85[ab]	26.14 ± 4.36[a]	57.13 ± 10.26[b]
DAB I (%)	67.00 ± 6.85	63.88 ± 6.57	62.50 ± 8.75	61.50 ± 6.36
DAB II	23.38 ± 4.14	23.50 ± 2.68	23.00 ± 3.49	25.50 ± 4.23
DAB III (%)	5.88 ± 1.91	8.50 ± 3.10	7.00 ± 2.24	9.75 ± 2.45
DAB IV (%)	3.75 ± 1.75	2.00 ± 0.82	3.00 ± 1.44	3.38 ± 1.56
HIGH MMP (%)	2.19 ± 0.90	1.79 ± 0.44	3.95 ± 1.04	2.70 ± 0.72
INTER MMP (%)	4.24 ± 0.98[a]	5.09 ± 1.18[ab]	10.12 ± 2.88[b]	8.34 ± 1.76[ab]
LOW MMP (%)	93.92 ± 1.80[a]	93.63 ± 1.17[ab]	86.70 ± 2.95[b]	89.68 ± 1.76[b]
TBARS (ng/10^6 sptz)	107.59 ± 31.54[a]	39.48 ± 19.26[b]	6.81 ± 3.06[c]	40.17 ± 6.56[ab]
CELLROX (%)	39.30 ± 1.39	41.67 ± 3.39	37.11 ± 1.83	35.95 ± 1.16
DCFH (%)	14.90 ± 7.94	7.07 ± 2.56	6.73 ± 1.07	14.82 ± 4.78
SCSA (%)	1.31 ± 0.43[a]	1.13 ± 0.43[ab]	0.39 ± 0.14[b]	0.84 ± 0.23[ab]
DMDA (%)	47.93 ± 7.91	49.58 ± 6.95	46.06 ± 8.39	50.53 ± 5.58
IMDA (%)	1.28 ± 0.22	1.37 ± 0.28	1.28 ± 0.28	1.14 ± 0.24
DMIA (%)	39.05 ± 5.74	38.41 ± 4.46	36.56 ± 5.03	38.04 ± 3.70
IMIA (%)	11.75 ± 3.02	10.65 ± 2.48	16.13 ± 6.20	10.32 ± 2.80

6 CONCLUSION

In conclusion, we observed that the glycolytic pathway is as important as oxidative phosphorylation for motility and ram sperm functionality. On the other hand, oxidative phosphorylation seems to have more influence in the sperm movement patterns than motility. In addition, we verified that the glycolytic pathway, after stimulation, is able to maintain sperm kinetic patterns, ATP levels and oxidative homeostasis of bovine epididymal spermatozoa submitted to mitochondrial uncoupling. Furthermore, we observed that the mitochondrial uncoupling associated with the glycolysis stimulation during the ovine sperm cryopreservation prevents oxidative injuries and then improving the post-thawing sperm quality.

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