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Cell bioenergetics and ATP production of boar spermatozoa

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ABSTRACT

Cellular metabolism is an important feature of spermatozoa that deserves more insights to be fully understood, in particular in porcine semen physiology. The present study aims to characterize the balance between glycolytic and oxidative metabolism in boar sperm cells. Agilent Seahorse technology was used to assess both oxygen consumption rate (OCR), as an oxidative metabolism index, and extracellular acidification rate (ECAR), as a glycolytic index. Different metabolic parameters were studied on freshly ejaculated sperm cells (identified as day zero sample, d0) and after one day of storage at 17 °C in Androhep extender (d1). Mitochondrial ATP production rate (MitoATP) was higher than the glycolytic ATP production rate (glycoATP) at both d0 and d1 while at d1 the amount of ATP production decreased, in particular, due to OXPHOS reduction. Conversely, glycoATP was not significantly different between d0 and d1. Interestingly, OCR profile showed no different bioenergetic parameters (*i.e.* ATP turnover, basal or maximal respiration, and spare respiration) between d0 and d1, thus indicating that sperm cell metabolism was reversibly decreased by preservation conditions. Other metabolic parameters showed the same trend, irrespective of the storage time: under stressed conditions (oligomycin plus FCCP), spermatozoa showed an increase in mitochondrial respiration while the metabolic potential of glycolysis did not undergo variations when compared to baseline metabolism. The rate of oxidation of fuel substrates – glucose, fatty acids, and glutamine – showed that sperm reliance on glucose oxidation to maintain baseline respiration was higher than fatty acids or glutamine. Interestingly spermatozoa demonstrated to have a low "capacity" parameter, which indicates that they cannot use only a single fuel substrate to produce energy. This feature of sperm metabolism to be unable to increase oxidation of a particular fuel to compensate for inhibition of alternative fuel pathway(s) was demonstrated by the negative value of "flexibility". Our results showed that ATP production in boar sperm cells relied on mitochondrial oxidative metabolism in freshly ejaculated cells, while, under liquid storage conditions, their oxidative metabolism decreased while the glycolysis remained constant. These results open new fields of research in the preservation techniques of boar sperm cells.

1. Introduction

Reproductive physiology of mammalian species is a scientific field in continuous evolution that pushes researchers to discover or uncover new features and insights into animal nature. In the physiology of male reproduction, in the last decades, several studies focused on sperm metabolism [1,2] giving important insights on the sperm function and energy management; additionally, "omics" [3] have become progressively more important and impacting on the understanding of physiological processes by adding new information and possibility to integrate them. A renewed interest on metabolism of sperm cells is taking place, as a better understanding of these aspects may help to greatly improve sperm preservation strategies, as well as their energy balance during capacitation and fertilization processes in many mammalian species [4–7].

Boar sperm cells have been studied by different approaches to determine their metabolic rate: metabolomics approach, using mass spectrometry to determine Glucose C^{13} and its metabolites, [8]; glycolysis pathways enzymatic activity study [9,10] or by specific pathway inhibition (*i.e.* using specific mitochondrial chain complexes inhibitors) and polarographical determination of oxygen consumption rate under defined conditions [11]. Given this quite broad amount of studies, there is still uncertainty regarding the effective pattern used by boar sperm cells to obtain energy: a general statement, assumed as the

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most probable, affirms that boar sperm cells principally rely on glycolysis for energy obtainment and cell function (for a thorough review, see Ref. [12]). In particular, a study on metabolomics of boar sperm cells [8] showed that boar sperm cells, use the glucose present in the medium pre-eminently by the glycolytic pathway, thus forming, as a metabolic by-product, lactate; the latter was demonstrated to be possibly transformed in pyruvate, apparently with a low activity of the Krebs cycle.

In a recent research from our group [11] we focused on mitochondrial activity in freshly ejaculated boar spermatozoa demonstrating that they can use oxidative metabolism when properly stimulated, as shown by the oxygen consumption rate (OCR) experiment. In addition, boar sperm mitochondria are coupled (ATP production depleted by oligomycin and maximal respiration rate enhanced by FCCP) and primarily use complex I instead of complex II to drive cell respiration and build the mitochondrial membrane potential [11].

The ability of boar sperm cells to metabolize other carbon-based molecules such as pyruvate or lactate (that are natural by-products of the glycolytic pathway) was demonstrated in early studies on sperm metabolism in 1973 [13], both under aerobic and anaerobic conditions. In particular, those authors demonstrated that the rate of pyruvate consumption is higher aerobically and that some by-products such as succinate and acetate are readily disappearing under aerobic conditions, thus showing an active aerobic metabolism in boar sperm cells. In more recent years, Medrano and colleagues [14] showed that boar spermatozoa can metabolize citrate and lactate via the LDH activity and Krebs cycle involvement, respectively.

Finally, the involvement of mitochondrial activity in the activation and functions of sperm cells has been demonstrated [15–17].

Given this background, the aim of the present study was to delineate which metabolic pathway is primarily involved in boar sperm energy obtainment thus using an experimental design that permits to assess glycolysis and oxidative phosphorylation at the same time both in freshly ejaculated (d0) and liquid-stored (17 °C) sperm cells for one day (d1).

2. Material and methods

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Boar semen was purchased from a commercial company (Inseme S.P.A., Modena, Italy). Semen collected from pure breed used for DOP production in Italy (large white, duroc and landrance) was resuspended in-farm in commercial extender (IMV Nutrixell+) and delivered within 2 h to the laboratory into a polystyrene box at room temperature; once in the lab, samples were pooled (three different ejaculates from different animals), washed and resuspended in Androhep medium [18] at a final concentration of 30×10^6 spz/mL. Minimum requirement for inclusion of sperm sample in the study was viability above 80% at the arrival at the laboratory.

A total of 15 ejaculates from 10 animals (aged from 10 months to 2 years) were used (5 replicates with 3 ejaculates pooled each repetition).

2.1. Cell metabolism analysis

The study of cellular metabolism was carried out by simultaneously measuring the rate of oxygen consumption (OCR), a cellular respiration index (pmol/min) and the rate of extracellular acidification (ECAR), a glycolysis index (mpH/min), using the Seahorse XFp analyzer (Agilent). The cells under study were freshly ejaculated boar sperm cells on the same day of collection (d0) and liquid-stored sperm cells after one day of storage at 17 °C (d1) diluted in Androhep extender.

1.5×10^6 cells were added in XFp (Agilent) cell culture microplates to each well previously coated with 10 μL of fibronectin (1 mg/mL in water) dried in an incubator at 37 °C for 2 h. Next, the plates were centrifuged at 1200×*g* for 1 min at 20 °C and the resulting supernatant was removed and replaced with 180 μL of Tyrode's medium (131.89 mM NaCl, 2.68 mM KCl, 1.80 mM $CaCl_2$, 0.19 mM $MgCl_2$, 0.36 mM KH_2PO_4 and 20 mM HEPES - default buffer factor, which takes into account the quantity of H^+ added to the analysis medium to modify the pH level by 1 unit of pH, was 2.6 nmol/L/pH) plus 5.56 mM Glucose, 2 mM L-glutamine and 1 mM sodium pyruvate, preheated 10 min at 37 °C; the analysis was thereafter started.

The injection ports of the XFp sensor cartridges, which were hydrated overnight with the XF calibrant at 37 °C, were loaded with a 10-fold concentration of modulators according to instructions provided by Seahorse XFp ATP Rate Assay, Cell Mito Stress Test, Cell Energy Phenotype Test and Mito Fuel Flex Test. All analyzes were performed at 37 °C. All data were analyzed by the WAVE software. Before starting the analyses, the OCR and ECAR values were normalized to 1×10^6 live cells depending on sperm cell viability.

2.2. Evaluation of ATP production

Cellular metabolic regulation allows cells to adapt to changes in ATP demand by responding to changes in ATP production to maintain total intracellular levels. A highly informative measure to describe cellular metabolism is the rate of ATP production, as it is the main dominant high-energy molecule in cells. Agilent Seahorse XFp Real-time ATP Rate assay measures the total rate of ATP production in living cells by distinguishing between ATP fractions produced by mitochondrial OXPHOS (mitoATP Production Rate) and glycolysis related to the conversion of glucose to lactate (glycoATP Production Rate), the two main metabolic pathways responsible to produce ATP in mammalian cells. The relationship between the mitoATP production rate and the glycoATP production rate (ATP Rate Index) allows us to detect changes in the metabolic phenotype (a ratio >1 mainly indicates the OXPHOS pathway; a ratio <1 means mainly glycolytic pathway). The assay consists of the use of metabolic modulators such as 1.5 μM oligomycin and a mixture of 0.5 μM rotenone and antimycin A, which, when injected in series, allow the calculation of the production rates of mitochondrial and glycolytic ATP providing a real-time measurement of the rates of cellular ATP production and a quantitative phenotype of cellular energy balance.

2.3. Measurement of the parameters of mitochondrial functions

Mitochondrial parameters were evaluated using the Mito Stress Test which consists of the use of metabolic modulators such as 1.5 μM oligomycin, 4.0 μM of Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) and a mixture of 0.5 μM rotenone and antimycin A, which are injected in series. The detected parameters are basal respiration (basal OCR before adding oligomycin), minimal respiration (OCR in the presence of oligomycin), maximal respiration (OCR after adding FCCP), proton leak (difference between basal respiration and respiration in the presence of oligomycin) indicating the translocation of H^+ into the intermembrane space independently of F_1F_O-ATP synthase. All parameters were subtracted from non-mitochondrial respiration and evaluated as OCR in the presence of rotenone and antimycin A. The difference between maximal and basal respiration provides the spare respiratory capacity, i.e. the cell's ability to respond to an increase in energy demand, considered a measure of the flexibility of the OXPHOS.

2.4. Cell energy phenotype

The cellular energy phenotype test rapidly measures mitochondrial respiration and glycolysis under basal and stressful conditions, to reveal the three key parameters of cellular energy metabolism: basal phenotype, stressed phenotype, and metabolic potential. By injecting 1.0 μM oligomycin and 4.0 μM FCCP at the same time, the tool allows us to evaluate two main parameters of cellular energy metabolism, known as metabolic phenotypes (basal and stressed phenotype) and metabolic potential. The basal phenotype is characterized by the OCR and ECAR values in the cells in the initial condition in the presence of substrates.

The stressed phenotype is shown by the OCR and ECAR values in the cells after the addition of stressful compounds (oligomycin and FCCP). The metabolic potential is the ability to increase energy production through respiration and glycolysis and is defined as the % increase in the stressed phenotype over the baseline phenotype of OCR and ECAR.

2.5. Oxidation of substrates

The Fuel Flex Test is a method of measuring mitochondrial fuel consumption in living cells for 80 min in which a decrease in oxygen consumption mirrors a decrease in fuel oxidation. Specifically, it determines the oxidation rate of glucose, fatty acids and glutamine by measuring the OCR. The parameters of cell dependency, capacity, and flexibility to oxidize the three mitochondrial fuels are evaluated by blocking the oxidation pathways of nutrients with specific inhibitors. By sequentially inhibiting the path of interest, followed by the two alternative pathways, it is shown how cells exploit the path of interest to satisfy their energy demand. The modulators used are: 3 μM BPTES (bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide), an allosteric inhibitor of glutamine oxidase, which blocks the oxidation pathway of glutamine, 4 μM etomoxir, the long-chain fatty acid pathway inhibitor, which inhibits the gene encoding carnitine palmitoyl-transferase 1 A and 2 μM UK5099, which inhibits glucose oxidation by blocking the mitochondrial vector of pyruvate. Fuel dependency represents the dependence of cells on a claimed oxidative pathway to maintain basic respiration. It is calculated by adding the specific inhibitor to block the pathway of interest followed by the inhibitors of two alternative pathways. Conversely, fuel capacity is shown by OCR driven by the specific oxidation of the substrate, achieved by blocking other fuel pathways. Fuel flexibility was achieved by subtracting fuel dependence from fuel capacity, for each path studied; this parameter describes the mitochondrial ability to switch from one oxidative pathway to another to allow the maintenance of OCR when a specific pathway is inhibited. Boar sperm might utilize oleic acid and palmitic acid as energy substrates for ATP production via β-oxidation [19], but the experiments were performed without the addition of exogenous fatty acids.

2.6. Viability assessment

For each pooled sample analyzed with the Seahorese® technology, an aliquot (25 μL) of semen (30×10^6 spz/mL) was incubated with 2 μL of a 300 mM propidium iodide (PI) stock solution (final concentration 16.7 mM), and 2 μL of a 10 mM SYBR green- 14 stock solution (final concentration 0.56 mM) live/dead sperm viability kit (Molecular Probes, Inc.) Cells were incubated 5 min at 37 °C in the dark. Ten μL of the sperm suspension were then placed on a slide and at least 200 spermatozoa per sample were scored using a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands). Spermatozoa stained with SYBR-14 but not with PI were considered viable (SYBR-14+/PI-).

2.7. Statistical analysis

The data represent the mean ± SD (shown as error bars in the figures); each experiment was performed five times. In each experimental set, the analyses were carried out on different pools of animals. The differences between data were evaluated by repeated measures ANOVA followed by Dunnet's test when F values indicated significance ($P \leq 0.05$).

3. Results

3.1. Boar sperm ATP production

The ATP synthesis by OXPHOS or glycolysis has been characterized by ATP production rate measurement related to the oxygen consumption in mitochondria (mitoATP production rate) and the conversion of glucose to lactate in the glycolytic pathway (glycoATP production rate) (Fig. 1A). The ATP production rate was detected on spermatozoa at d0 or d1 by injecting oligomycin, to inhibit mitochondrial ATP synthesis and then rotenone plus antimycin A to block mitochondrial respiration. We obtained the mitoATP production and the response of glycolysis in the cell by glycoATP production. The results highlighted the feature of sperm cells characterized by oxidative metabolism. In particular, the mitoATP production rate at d1 was three times lower than at d0. This time-dependent ATP production did not affect glycolysis. Since mitoATP production corresponded to 85% of total ATP production (mitoATP + glycoATP) as a consequence the total ATP production at d1 was inhibited by 70%. Even if sperm cells decreased ATP production at d1, the ratio between mitoATP Production Rate and glycoATP Production Rate (ATP Rate Index) was always >1 and there were no significative differences between d0 and d1 (Fig. 1B). This metric for detecting changes and/or differences in the metabolic phenotype corroborated mainly the evidence of a more oxidative phenotype (OXPHOS pathway) in boar sperm cells.

3.2. Bioenergetic parameters of mitochondria sperm cells

By sequential use of mitochondrial modulators, i.e. oligomycin, FCCP, and rotenone plus antimycin A mixture, we obtained the cellular respiratory profile of sperm at d0 and d1 (Fig. 2A). We noticed an identical response to injected mitochondrial modulators regardless of spermatozoa preservation time. As a consequence, the OCR values at d0 were not different from those at d1. The different times of sperm cells storage did not change any mitochondrial parameters: basal respiration (detected as baseline OCR before oligomycin addition); minimal respiration (measured as OCR in the presence of oligomycin); maximal respiration (evaluated as OCR after FCCP addition); non-mitochondrial respiration (evaluated as OCR in the presence of rotenone plus antimycin A). The latter was subtracted from all the mitochondrial parameters (Fig. 2B). Moreover, we highlighted OCR values of basal respiration and ATP production that confirmed a satisfactory coupling efficiency in sperm cells of 0.92 ± 0.02 a.u. and 0.92 ± 0.07 a.u. at d0 and d1, respectively (the maximal value of 1.0 a.u. is obtained when all the basal respiration is sensitive to oligomycin). In addition to this, the spare respiratory capacity, which defines the cell propensity to adjust cell bioenergetics to fulfil the increased energy demand required by spermatozoa functions of motility, represented 52.7% and 53.9% of the maximal respiration at d0 and d1, respectively. These results were consistent with the striking increase in maximal respiration, evaluated after FCCP addition, which attained 2.1 and 2.2 higher values than basal respiration at d0 and at d1, respectively.

3.3. Metabolic phenotype of sperm cells

The phenogram profile of spermatozoa is depicted in Fig. 3A. Treatment of sperm cells with oligomycin and FCCP mixture, known as a mitochondrial stressor, provided the metabolic state of cells under the relative baseline and stressed conditions. Sperm cells switched to a state of energy demand increasing the OCR, whereas the ECAR were unaffected. Accordingly, the aerobic and the energetic states were increased (Fig. 3A). The metabolic potential response (expressed as increased OCR or ECAR over baseline %) showed a greater utilization only of spermatozoa mitochondrial respiration (stressed OCR) increasing approximately 2.5 times in the same way at d0 and d1 (Fig. 3B). Conversely, the glycolytic metabolic potential (stressed ECAR) remained at baseline in both cases (Fig. 3B). Therefore, the spermatozoa can respond to ATP depletion with an increase in respiration by mitochondrial respiration but not with glycolysis.

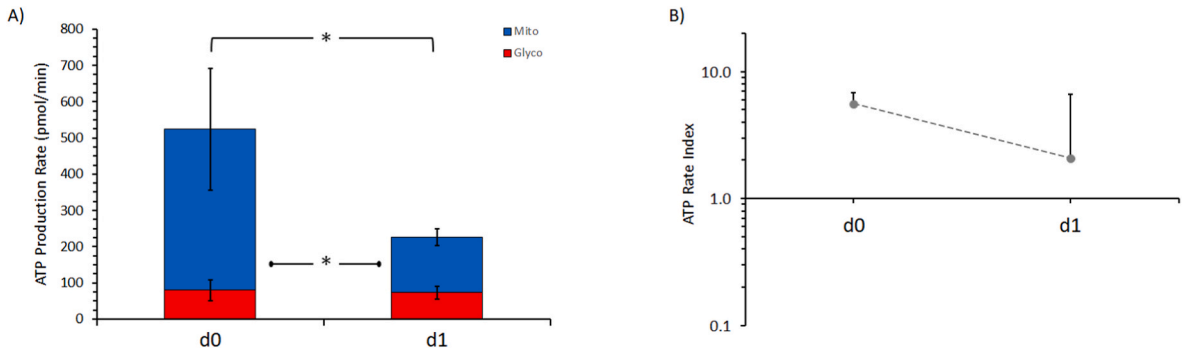

Fig. 1. Profile of ATP production in boar spermatozoa. A) Evaluation of ATP production rate by mitochondrial OXPHOS (blue bars) or by glycolysis (red bars) on spermatozoa at 0 day (d0) and 1 day (d1). B) The ATP rate index, calculated as the ratio between the mitochondrial ATP production rate and the glycolytic ATP production rate, is shown on the *y*-axis (logarithmic scale) in spermatozoa at d0 and d1. Data represent the mean ± SD (vertical bars) from five experiments carried out on distinct cell preparations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

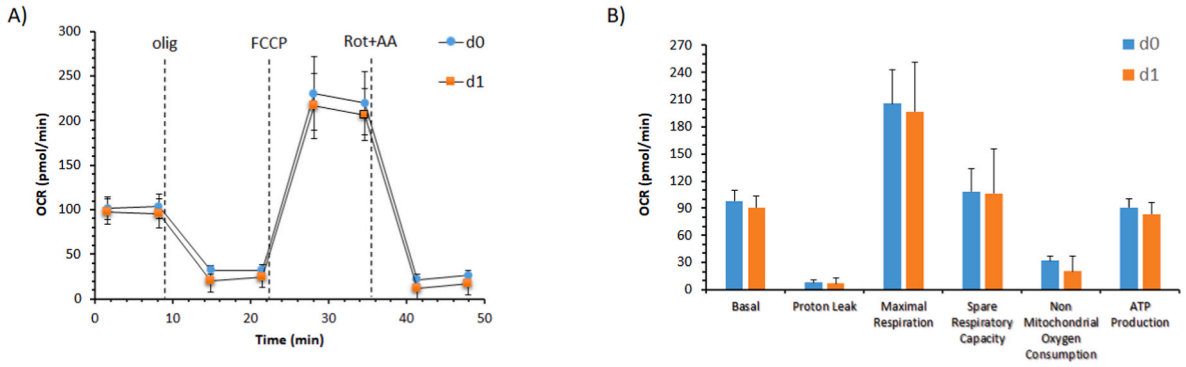

Fig. 2. Sperm cells respiration. A) Profile of oxygen consumption rate (OCR) 0 day (d0, blue) and 1 day (d1, orange) under basal respiration conditions and after the addition of 1.5 μM oligomycin (olig), 1.0 μM FCCP and a mixture of 0.5 μM rotenone plus antimycin A (rot + AA). B) Mitochondrial parameters (basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial oxygen consumption, ATP production) of spermatozoa at d0 (blue) and d1 (orange). Data represent the mean ± SD (vertical bars) from five experiments carried out on distinct cell preparations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

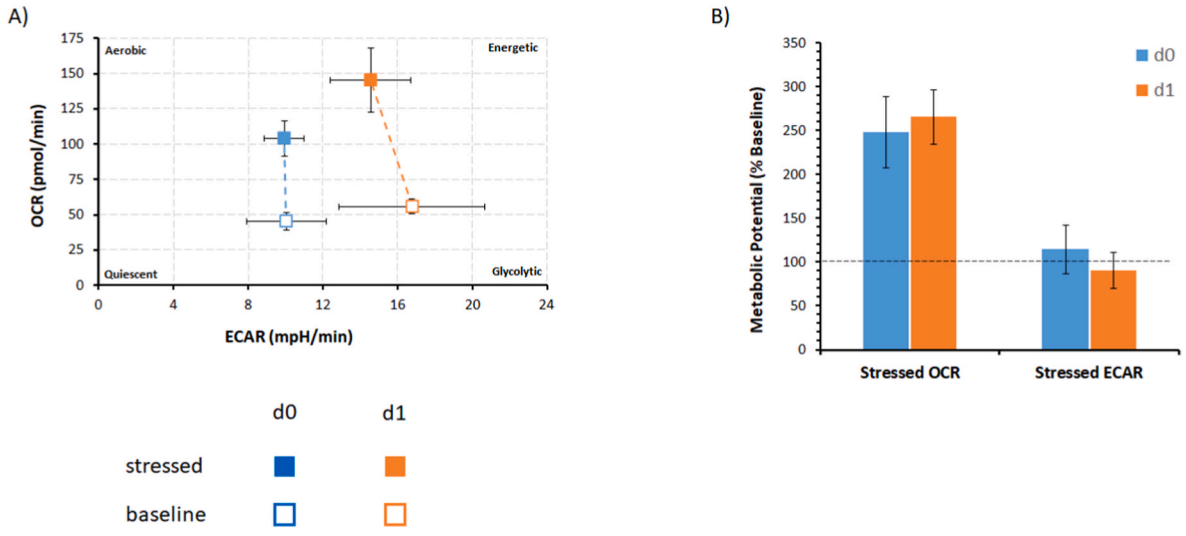

Fig. 3. Metabolic switching of spermatozoa. A) Baseline phenotype of 0 day (d0) (open blue square) and 1 day (d1) spermatozoa (open orange square) and stressed phenotype of d0 (closed blue square) and d1 spermatozoa (closed orange square). B) Metabolic potential in "Stressed OCR" and "Stressed ECAR" is expressed as % "Baseline OCR" and "Baseline ECAR" (dashed horizontal line) at d0 (blue bars) and d1 (orange bars). Data represent the mean ± SD (vertical and where present also horizontal bars) from five experiments carried out on distinct cell preparations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Substrate oxidation in sperm cells

The time-dependent ATP production in spermatozoa has been evaluated regarding the preferred three mitochondrial fuels (glucose, glutamine, and fatty acids) utilization for cell respiration (Fig. 4). The inhibition of the oxidative pathway of each substrate ensured the calculation of the parameters of fuel dependency, capacity, and flexibility of sperm cells. By measurement of the mitochondrial respiration has been determined the rate of oxidation of each fuel. In particular, sperm cells' mitochondria did not show a difference in fuel pathways at d0 or d1. Glucose was the substrate with a higher dependency than other fuels. However, the negative value of flexibility for all the substrates procured a lower capacity value than dependency (Fig. 4). In other words, the mitochondria of spermatozoa required that glucose, glutamine, and fatty acids were oxidate in the fuel pathways to maintain basal cell respiration.

4. Discussion

Spermatozoa are motile cells, and this has been the main characteristics observed by scientists since van Leeuwenhoekin first discovered these "animalicules" in the late 1600s and then Spallanzani published on this tocpic in 1784. Subsequently, the interest in sperm cells focused on the metabolic pathway to energy obtainment as was reported and thoroughly described in Ref. [4]. Other experimental evidence [8,9,14,20] lead the researchers to define boar spermatozoa as primarily glycolytic cells with low or no capacity to perform gluconeogenesis and very low glicogneosyhtetic activity. On the other side, evidence of lactate/pyruvate metabolization [13,14,20] and the activity of electron transport chain activity in ATP production [11,21] clearly demonstrate the intervention of mitochondria in energy obtainment by sperm cells.

This scenario apparently leaves some open questions: is OXPHOS always active in boar sperm cells? Do mitochondria play a pivotal role in energy-obtainment strategies in these cells?

Understanding the importance of energetic metabolism is essential to develop proper preservation strategies for sperm cells. It is known that porcine sperm storage at 16–17 °C induces a reduction of the metabolic activity of sperm cells [18], but no information regarding the actual balance of metabolic pathways has been demonstrated since now.

Results obtained in this study give interesting insights into the metabolism of spermatozoa in porcine species. At d0, freshly ejaculated semen showed a relatively high metabolic rate in ATP formation and, most interestingly, more than 80% of this ATP is formed by OHPHOS. These cells were ejaculated at a temperature near 36 °C, then diluted with a commercial extender and subsequently transported into the lab within 1 h. The analysis was carried out before they can experience cooling to 17 °C, therefore no decrease in metabolic activity is expected. A different situation is reflected after one-day storage, d1, when these cells may encounter metabolic rewiring that changes demands for homeostasis and motility [4]. In fact, d1 spermatozoa (after preservation at 17 °C) showed a significant decrease in ATP production, in particular due to a decrease in OXPHOS, even if no alteration in anaerobic production by glycolysis was displayed. These data are important as they clearly demonstrate what was not yet experimentally evidenced in other publications, that is to say that sperm metabolism during preservation actually decreases, especially mitochondrial one; in addition, our findings furnish an interesting hint of discussion about data from other researchers, who described a pre-eminent glycolytic metabolism [8]. It is possible that cells analyzed by those Authors had been preserved at 17 °C for a longer time compared to what we performed, or, at least, being commercial doses, they underwent a cooling process at 17 °C and that, as a consequence, the glycol/mito ATP production ratio underwent a shift from what we observed. Those Authors, in a recent publication [21] demonstrated that preserved boar semen can resume a very high mitochondrial activity after proper stimulation with red led light, as they noticed a very high OCR determined with a different method than the one we used (SensorDish® Reader (SDR) system).

Moreover, from a bioenergetic point of view, the importance of mitochondria as the powerhouse of spermatozoa was corroborated by the results on coupling efficiency of mitochondria in sperm cells near the maximal value of 1.0 a.u., highlighting the important role of mitochondria in spermatozoa to synthesize ATP.

Interestingly, the decrease in mitochondrial ATP production at d1 did not alter the oxidative metabolic profile of sperm cells, as sperm cells' respiration profile at d0 and d1 and the key parameters of cell respiration analyzed were not different (as demonstrated by the mito-stress test). This test is based on a stimulation of the mitochondria by "stressing" their components (various complexes) and its results suggest that ATP decrease at d1, due to a decreased ATP production from mitochondria, is not attributable to mitochondrial dysfunction, but it is the effect of proper preservation condition, decreasing metabolic activity in a reversible manner. Consistently, mitochondria preserved the ability to increase the performance in energy production under stress conditions, which required an increase in ATP synthesis (as demonstrated by the results of the cellular energy phenotype test). This feature was not owned by glycolysis. This finding gives interesting indications on the metabolic strategy of boar spermatozoa in energetically demanding situations of their lives, such as capacitation. Indeed, it is reasonable to suppose that the main energetic pathway involved in supporting capacitation and sperm cells' hyperactivation would be OXPHOS. Recent preliminar results from our lab (data not shown), seem to support this hypothesis. Previous studies have also reported that mitochondrial activity is required for sperm cells to undergo the capacitation process *in vitro* [15–17].

We can speculate that the metabolism of sperm cells could change by relying on different substrates to support cellular energy demands after one day. We noticed that sperm cells probably prefer glucose to glutamine or fatty acids as the main substrate given that the dependency value was the highest. Nevertheless, this feature remained similar at d0 and d1. Despite this, dependency indicated that the spermatozoa's mitochondria were unable to compensate for the blocked glucose oxidation pathway by oxidizing glutamine and fatty acids. The presence of a negative value of flexibility indicated the cells' mitochondria could not compensate for the inhibited pathway by utilizing other pathways to fuel mitochondrial respiration at d0 or d1. The presence of dependency and absence of flexibility (negative values in all three substrates tested) demonstrated that the mitochondria require glucose, glutamine, and fatty acid pathways to maintain basal cell respiration.

The overall picture of boar sperm cells' metabolism is at least enriched by our results: carbohydrates play an important role in supporting the fueling machinery of energetic metabolism [22,23] but they are not only metabolized through an anaerobic pathway, which results, indeed, the less active if compared to the oxidative phosphorylation pathway. We can finally assert that boar sperm cells use preferentially the oxidative phosphorylation pathway, in particular through complex I instead of complex II [11], and that they have the ability to enhance ATP production by OXPHOS and to resume mitochondrial function after preservation under cooled conditions.

It should be stressed that recent studies in mouse spermatozoa, regarded for decades as strictly glycolytic cells, [24], showed that these cells actually present an active OXPHOS activity [25] that sustains motility: in this respect, our findings are precious to better re-define the main sperm metabolic pathways used by boar spermatozoa.

These findings give important indications on the formulation of extenders for boar preservation, to enhance their capacity to perform at best under demanding conditions; in addition, this metabolic study approach can be applied also to understand the physiology of spermatozoa during capacitation or the possible effect of different substances (antioxidants, xenobiotics) on sperm mitochondrial function and metabolism.

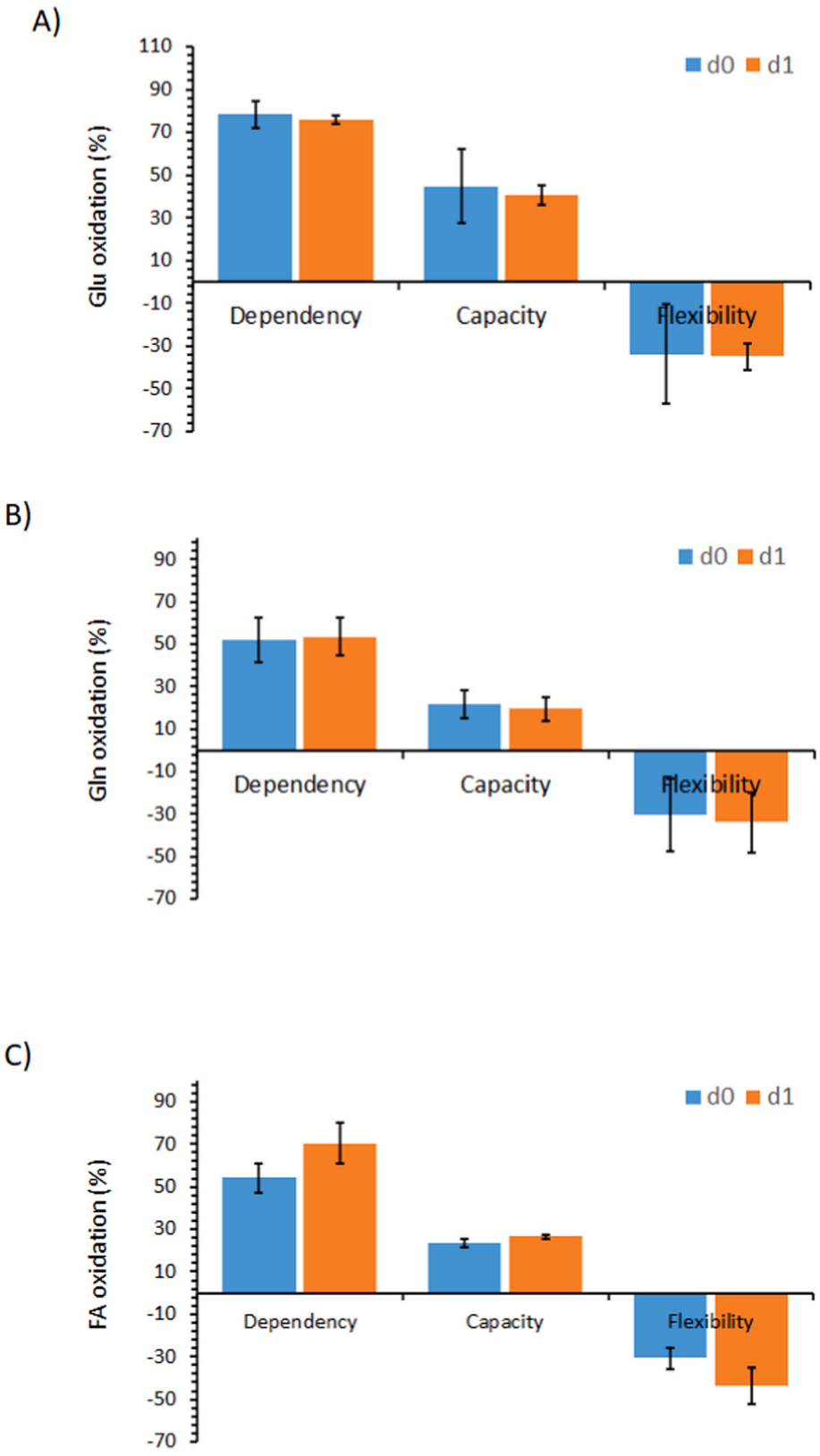

Fig. 4. Determination of the oxidation rate of fuels. Fuel dependency, capacity and flexibility are calculated on the oxidation of glucose (A), glutamine (B), and fatty acids (C) at 0 day (d0, blue) and 1 day (d1, orange). The histogram represents the mean ± SD (vertical bars) of five experiments conducted on different cell preparations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CRediT authorship contribution statement

Olga Blanco Prieto: Investigation. **Cristina Algieri:** Investigation. **Marcella Spinaci:** Validation, Visualization, Supervision, Writing – review & editing. **Fabiana Trombetti:** Validation, Writing – review & editing. **Salvatore Nesci:** Conceptualization, Formal analysis, Visualization, Supervision, Writing – original draft. **Diego Bucci:** Conceptualization, Formal analysis, Visualization, Supervision, Writing – original draft, Funding acquisition.

Aknowledments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.07.018.

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