Animal Reproduction Science 278 (2025) 107875

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Mitochondrial bioenergetics analysis on SLC-selected boar spermatozoa during liquid storage

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ARTICLE INFO

Keywords:
Porcine spermatozoa
Colloid
SLC
Energy metabolism
Agilent Seahorse®
ATP production

ABSTRACT

Semen preservation at 17 °C plays a pivotal role in the porcine breeding industry, though it presents challenges; not all ejaculates retain their quality during storage. Colloidal centrifugation has been developed to improve it since this method eliminates seminal plasma and microorganisms while simultaneously allowing the selection of spermatozoa based on gradient density centrifugation. This study aimed to evaluate whether two different colloid densities (1.026 g/ml – 20 %Porcicoll® and 1.104 g/ml – 80 % Porcicoll®) improve sperm bioenergetics, assessed using Agilent seahorse, along with key semen quality parameters such as viability, mitochondrial activity, oxidative stress and motility analyzed through flow cytometry and the CASA system over time. Fifteen ejaculates were divided into three groups: Control (CTR), 20 % Porcicoll® (P20), and 80 % Porcicoll® (P80). Sperm parameters were analyzed at days 0, 3, and 7 of storage. Results revealed a metabolic shift from mitochondrial oxidative phosphorylation to glycolysis during storage, with a 15 % decrease in ATP production by day 3. P80-treated sperm showed higher ATP production on day 0 compared to P20. Sperm quality parameters such as viability and mitochondrial activity decreased after colloidal centrifugation, possibly because of early capacitation; we also hypothesize that SLC could increase sensitivity to the high glucose levels in the medium. Although Porcicoll® effectively selected metabolically active sperm, the lack of seminal plasma and limitations in media composition negatively affected long-term sperm quality. Further research is necessary to optimize media formulations and assess fertility outcomes of selected sperm for enhanced sperm preservation techniques.

1. Introduction

Boar semen storage at 17 °C is the most used technique to preserve porcine semen for artificial insemination (AI) (Knox, 2016; Mellagi et al., 2023; Waberski et al., 2019). Preservation techniques are a critical point in the AI industry since they allow semen to be stored for several days (up to seven) with almost no loss in quality and fertility parameters; however, this long-lasting preservation

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https://doi.org/10.1016/j.anireprosci.2025.107875
Received 22 October 2024; Received in revised form 21 May 2025; Accepted 29 May 2025
Available online 29 May 2025

raises concerns about bacterial contamination and control of bacterial growth, as reported in several articles (Althouse et al., 2000; Althouse and Lu, 2005; Kuster and Althouse, 2016; Maroto Martín et al., 2010). The vast majority of bacteria found in fresh ejaculates are Gram-negative and come from the Enterobacteriaceae family, originating from animal and non-animal sources (Althouse and Lu, 2005; Dalmutt et al., 2020; Úbeda et al., 2013). Semen bacteria proliferation can modify the characteristics of the extenders and directly affect sperm quality (Martínez-Pastor et al., 2021; Ngo et al., 2023). In addition, the spread of antibiotic resistance in inseminated females and workers of the pig industry emphasizes the importance of controlling the spread of antimicrobial resistance among bacteria (Kellerman et al., 2022; Nitsche-Melkus et al., 2020; Van Boeckel et al., 2019). Antibiotics should be strictly limited to therapeutic purposes only when necessary and should, therefore, be used preferably after determining the appropriate molecule regarding pharmacokinetics and specificity for bacteria.

Recent studies have focused on physical solutions to overcome this problem. The most studied and practical is density gradient centrifugation, specifically, single layer centrifugation (SLC) (Lacalle, Fernández-Alegre, et al., 2023; Lacalle, Martínez-Martínez, et al., 2023; Macías García et al., 2009; Martínez-Pastor et al., 2021; J. Morrell et al., 2009; J. M. Morrell et al., 2019). This technique, in particular in boar using the Porcicoll® colloid (Crespo-Félez et al., 2017; Deori et al., 2020; Martinez-Alborcia et al., 2013), has demonstrated its value in removing bacteria from semen preparations in several species (Cojkic et al., 2024; Guimarães et al., 2015; Luño et al., 2020; J. M. Morrell et al., 2014, 2019; J. M. Morrell, Saravia, et al., 2009; J. M. Morrell and Wallgren, 2011b; Varela Fernández et al., 2018), also allowing selection of the best spermatozoa in terms of different characteristics (Al-Kass et al., 2019; Bucci et al., 2013; Lacalle, Fernández-Alegre, et al., 2023; Lima-Verde et al., 2022; Martinez-Alborcia et al., 2013; Šterbenc et al., 2019; Yulnawati et al., 2014). The technique is practical in some scenarios for processing boar semen, in particular because of its scalability (Lacalle, Fernández-Alegre, et al., 2023; Martinez-Alborcia et al., 2013; Martínez-Pastor et al., 2021; van Wienen et al., 2011), resulting in the selection of sperm with superior characteristics (Martínez-Pastor et al., 2021; J. M. Morrell, Saravia, et al., 2009; J. M. Morrell and Wallgren, 2011a). It has been described that Porcicoll® at 40 % (1.052 g/ml) can maintain sperm quality during one-week storage at 16–18 °C (J. M. Morrell et al., 2019). Moreover, a low-density Porcicoll® colloid (20 %, 1.026 g/ml and 30 %, 1.039 g/ml) allows for the removal of seminal plasma and removes or significantly reduces microorganism load while recovering most spermatozoa (Lacalle, Fernández-Alegre, et al., 2023; Lacalle, Martínez-Martínez, et al., 2023), unlike higher-density Porcicoll®, which retains a considerable proportion of poor quality and dead spermatozoa.

Research on sperm metabolism has gained new importance in recent years after decades of scant interest (Bucci et al., 2011, 2022; Nesci et al., 2020) despite being very relevant for improving sperm storage. Pig spermatozoa, which rank among the most extensively studied, are known to differ from those of other species in the ways they acquire energy and the metabolic pathways they employ to sustain their motility and function. These pig sperm depend on mitochondrial oxidative phosphorylation as their main source of energy production, implying that mitochondria play a central role in ATP generation. Glycolysis is secondary in pig spermatozoa, contributing less significantly to overall energy production. This reduced glucose utilization could limit their capacity to adapt to environments with low oxygen availability -such as bacterial contamination- where bacteria and spermatozoa compete for nutrients and oxygen (Nesci et al., 2020). Moreover, the formulation of semen extenders aims to better preserve sperm cells from cold shock, oxidative stress, and ageing, thus maintaining the energy machinery intact. This allows the sperm cells to reach proper capacitation in the utero-tubal junction for the precise moment of the oocyte arrival or for in vitro fertilization (Prieto et al., 2023).

The Seahorse XF Analyzer system is a novel approach to evaluating energy metabolism. Although this technology was developed over the past decade, its use in more focused investigations—such as sperm metabolism—represents a more recent innovation. This method offers several advantages, including providing dynamic, real-time data on energy metabolism, the simultaneous assessment of multiple metabolic parameters, and the requirement of only small quantities of viable cells, which is highly beneficial when limited sample availability (Nesci et al., 2020).

Considering the positive effects of SLC using low-density Porcicoll®, our starting hypothesis is that boar sperm bioenergetics could be better preserved during refrigerated storage if semen is previously processed. Therefore, we designed a study with the objective of testing if SLC using two different colloid concentrations could improve sperm energetic parameters and their relationship with other sperm quality parameters (viability, mitochondrial activity, oxidative stress, CASA motility).

2. Material and methods

2.1. Experimental design

A total of 15 ejaculates from fertile boars were obtained from ANAS (Associazione nazionale allevatori suini, Rome, Italy) and used for the experiments. The boars were between 12 and 22 months old and kept under standardized environmental conditions: 18 and 23 °C temperature and light/darkness cycles (12 hours/12 hours). These boars were part of the center's routine production. They obtained the semen in their routine activity (three times every two weeks) and provided the ejaculates to the researchers without them contacting the animals nor affecting the collection routine.

Once in the laboratory, samples were centrifuged (900 ×g 2 min) and extended at 100×10^6 spermatozoa per ml (sperm/ml) in Androhep® (glucose monohydrate (26 g/l), sodium citrate (8 g/l), sodium bicarbonate (1.2 g/l), EDTA (disodium salt; 2.4 g/l), bovine serum albumin (BSA; 2.5 g/l), sodium penicillin G (0.6 g/l), dihydrostreptomycin (1 g/l). The colloid is a silane-coated silica formulation designed explicitly for boar semen (Porcicoll), prepared at densities of 1.026 g/ml (20 %, P20) and 1.104 g/ml (80 %, P80). The diluted semen was divided into an uncentrifuged control group (CTR), SLC with P80, and SLC with P20. SLC was prepared by layering 5 ml of extended semen over 5 ml of colloid in 10 ml centrifuge bottles, followed by centrifugation at 300 ×g for 20 min. The supernatant was removed using a water pump, and the pellet was resuspended in sterile Androhep® to a final volume of 5 ml. P20

separates spermatozoa from seminal plasma without selecting for specific cells, whereas P80 selects for the most robust spermatozoa, removing both dead and suboptimal spermatozoa and seminal plasma. The concentration of every sample was adjusted to 30×10^6 ml^{-1}, and the samples were stored at 17 °C for one week. At days 0, 3, and 7 of storage (D0, D3, and D7, respectively), the samples were analyzed for bioenergetics with Agilent Seahorse®, sperm motility by CASA, and sperm quality (viability, mitochondrial membrane potential, and mitochondrial ROS production) by flow cytometry.

2.2. Chemicals

Generic chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Flow cytometry reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.3. Sperm bioenergetics

2.3.1. Cell metabolism analysis

The study of cellular metabolism was carried out by simultaneously measuring the rate of oxygen consumption (OCR) and the rate of extracellular acidification (ECAR) using the Seahorse XFp analyzer (Agilent). OCR is a cellular respiration index (pmol/min), and ECAR is a glycolysis index (mpH/min). For analysis, 1.5×10^6 cells from each sample were added to individual wells in XFp (Agilent) cell culture microplates. Each well was previously coated with 10 μL of fibronectin (1 mg/ml in water) and dried in an incubator (37 °C, 2 h). After adding the samples, the plates were centrifuged (1200 ×g,1 min) at 20 °C; the supernatant was removed and replaced with 180 μL of Tyrode's medium at room temperature (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 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 started immediately after adding the medium and maintaining the temperature at 37 ºC.

The injection ports of the XFp sensor cartridges, which were hydrated overnight with XF calibrant at 37 °C, were loaded with a 10-fold concentration of modulators according to instructions provided by the Seahorse XFp ATP Rate Assay. All analyses were performed at 37 °C. All data were analyzed using the WAVE version 2.6.3 software, and the OCR and ECAR values were normalized to 1×10^6 live cells depending on sperm cell viability.

2.3.2. Evaluation of ATP production

Cellular metabolic regulation allows cells to adapt to changes in ATP demand by responding with changes in ATP production to maintain intracellular ATP levels. A highly informative measure to describe cellular metabolism is the rate of ATP production, as it is the primary high-energy molecule in cells. This rate was characterized by the ATP Rate Assay, which discriminates among the ATP produced in the glycolytic pathway (conversion of glucose to lactate, glycoATP production rate) and the mitochondrial oxidative phosphorylation (OxPhos) pathway (mitoATP production rate). The ratio between the mitoATP production rate and the glycoATP production rate (ATP rate index) is a useful parameter for detecting changes and/or differences in the metabolic phenotype. Thus, a ratio > 1 suggests a predominance of the OxPhos pathway; a ratio < 1 indicates a higher production from the glycolytic pathway. The assay uses metabolic modulators such as 1.5 μM oligomycin and a mixture of 0.5 μM rotenone and antimycin A; injected in series, these modulators allow the calculation of the production rates of mitochondrial and glycolytic ATP. This provides a real-time measurement of the rates of cellular ATP production and a quantitative definition of a phenotype of cellular energy balance (Algieri et al., 2023).

2.4. Motility assessment

Sperm motility was assessed with a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12). First, 5 μL of the sample was diluted in 10 μL of extender and prewarmed in a water bath (37 °C) for 5 min. Then, a 5-μL drop was put into a Makler counting chamber (20 μm depth; Sefi Medical Instruments, Haifa, Israel) and a phase contrast microscope (Nikon E400 with warmed stage at 37 °C; x10 negative contrast optics).

Three drops of each sample were evaluated, and at least 400 spermatozoa per sample and replicate were evaluated. The settings for the CASA system were as follows: 60 frames per second; minimum contrast at 49; minimum cell size 6 pixels; percentage of straightness: 75; static cell cut-off: VAP 20 μm/s and VSL 5 μm/s. Sperm motility endpoints were: total motile spermatozoa (TM, %); progressive spermatozoa (PM; %); curvilinear velocity (VCL, μm/s); average-path velocity (VAP, μm/s); straight-line velocity (VSL, μm/s); linearity (LIN); straightness (STR); wobble (WOB); lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

2.5. Flow cytometry analyses

Flow cytometry was conducted to evaluate sperm viability, mitochondrial activity (mitochondrial membrane potential), and mitochondrial reactive oxygen species (ROS; $O_2^{\bullet-}$). Analyses were performed following the recommendations of the International Society for Advancement of Cytometry (Lee et al., 2008). In each assay, sperm concentration was adjusted to 1×10^6 spz/ml in a final volume of 0.3 ml BTS media, and spermatozoa were stained with the combinations of fluorochromes (described below). Stained samples were passed through a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488 nm argon-ion laser and a 635 nm red diode laser. The emission of each fluorochrome was detected by using filters: 530/30 band-pass (green/FL1), 585/42 band-pass (orange/FL2), $>$ 670 long pass (far-red/FL3), and 661/16 band-pass (orange for red laser/FL4). Data were acquired using the BD CellQuest Pro software v. 5 (Becton Dickinson), and analysis was performed using Weasel v. 3.8.

Fluorescent probes were prepared at the following final concentrations: SYBR 14 (100 nM), SYTOX Green (1 nM), and propidium iodide (PI; 12 mM) for viability; monomeric JC-1 (8.3 μM) for mitochondrial membrane potential; and MitoSOX Red (1 μM) for mitochondrial ROS production.

Fluorescent signals were logarithmically amplified, and photomultiplier settings were adjusted for each staining method. FL1 was used to detect green fluorescence from SYBR 14, SYTOX Green, and monomeric JC-1; FL3 was used to detect the red fluorescence from PI and MX; FL2 was used to detect the orange fluorescence from JC-1 aggregates (active mitochondria).

A morphological dot plot was set by recording side scatter height (SSC-H) and forward scatter height (FSC-H) in linear mode (in FSC vs. SSC dot plots). The sperm population was positively gated based on FSC and SSC. Other events were gated out. A minimum of 10,000 gated sperm events was evaluated per replicate.

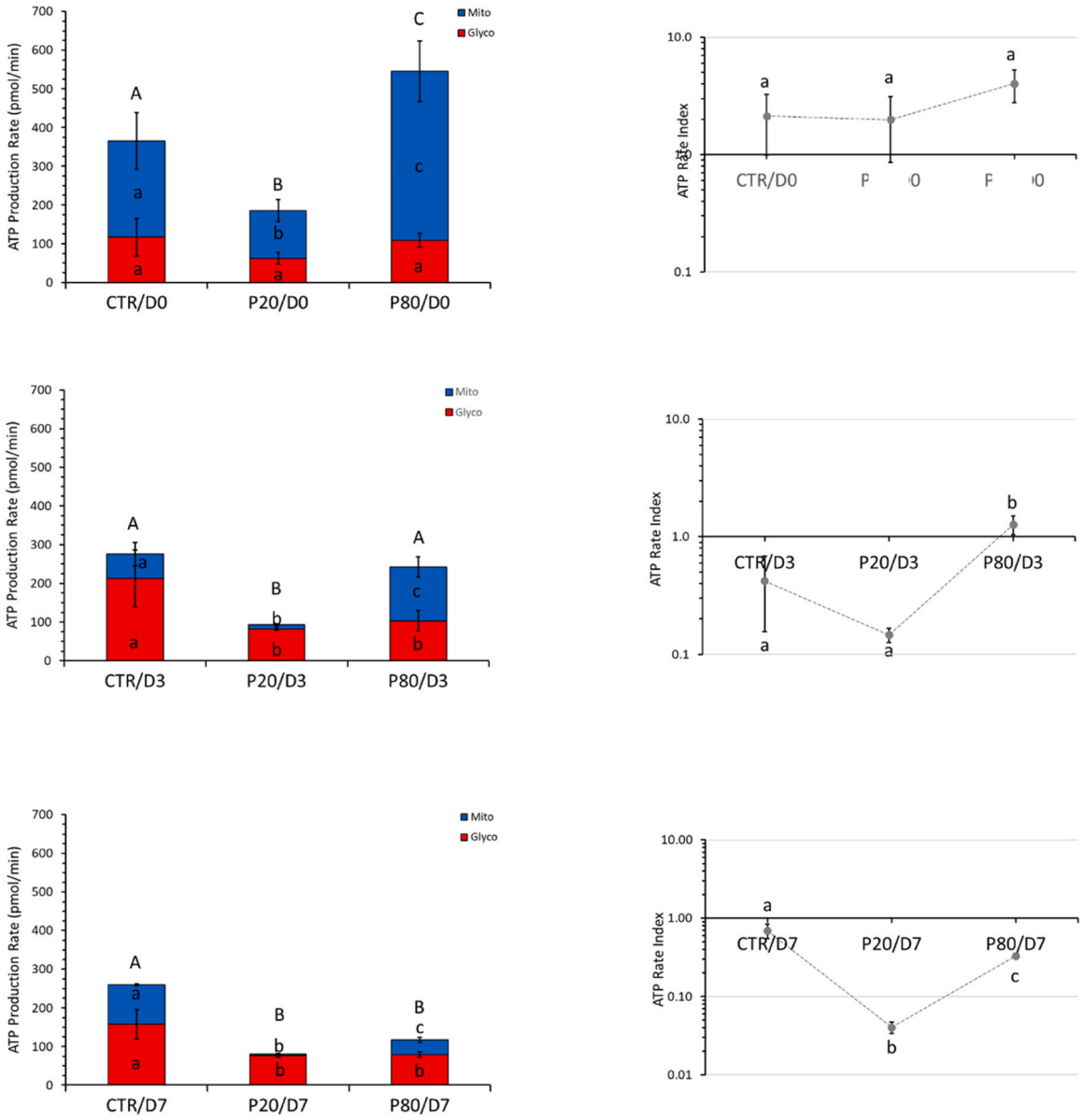

Fig. 1. Effect time-dependent (D0, D3 and D7) of 0 % (CTR), 20 % (P20) or 80 % (P80) colloid on the real-time ATP production rate in sperm cells from boars (n = 3). The evaluation of the ATP production rate by mitochondrial OxPhos is shown in blue and glycolysis in red. The ATP rate index, calculated as the ratio between the mitochondrial and glycolytic ATP production rates, is shown on the y-axis (logarithmic scale) in sperm cells. Data, expressed as a column chart and points, represents the mean ± SD (vertical bars) from three experiments on distinct cell preparations. Comparisons were carried out using Dunnet's test with Bonferroni correction. Different lower-case letters indicate significantly different values ($p < 0.05$) among treatments (CTR, P20, P80) in the same metabolic pathway; different upper-case letters indicate different values ($p < 0.05$) among treatments in ATP production rates resulting from the sum of OxPhos and glycolysis.

In MitoSOX-SYTOX Green flow cytometric assessments, percentages of non-DNA-containing particles (debris) were determined to prevent overestimating sperm particles in the lower-left quadrant (LL, full negative) using information from the SYBR 14/PI analysis, as described by Petrunkina et al. (2010).

2.5.1. Viability (SYBR-14/PI)

Sperm viability and debris interference in other stain combinations were determined by assessing the membrane integrity using the SYBR-14 and PI (L7011, ThermoFisher Scientific, Waltham, Massachusetts, EEUU) fluorochromes (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy). SYBR-14 is a membrane-permeable dye and stains the head of viable spermatozoa green. At the same time, PI is a membrane-impermeable dye that only penetrates through a disrupted plasma membrane, staining non-viable sperm heads red. Sperm were aliquoted in 300 μL stained samples with SYBR-14 and PI fluorochromes for 10 min at 37 °C in the dark. Viable spermatozoa exhibited positive staining for SYBR-14 and negative staining for PI (SYBR-14 +/PI-). In addition, as indicated before, the Live/Dead Sperm Viability kit is used to identify non-sperm particles in the lower-left quadrant (Petrunkina et al., 2010).

2.5.2. Mitochondrial ROS production (SYTOX-Green/MitoSOX Red)

MitoSOX Red (MX; M36008, ThermoFisher Scientific, Waltham, Massachusetts, EEUU) is a lipid-soluble, cell-permeable cation that selectively targets the mitochondrial matrix and thus can detect the generation of superoxide radicals ($O_2^{\bullet-}$) in this organelle. MX emits red fluorescence upon oxidation, detected by the FL3 detector. It was coupled with SYTOX Green (S7020, ThermoFisher Scientific, Waltham, Massachusetts, EEUU) as a viability counterstain. SYTOX Green stains dead cells and emits green fluorescence upon binding to DNA, detected by the FL1 detector. Sperm were aliquoted in 300 μL stained samples with SYTOX Green and MX and incubated at 37 °C for 30 min in the dark. Mitochondrial ROS production by viable cells was recorded.

2.5.3. Mitochondrial membrane potential assessment

JC-1 (T3168, ThermoFisher Scientific, Waltham, Massachusetts, EEUU) analysis was run to study mitochondrial membrane potential. Sperm were incubated with JC-1 at 37 °C for 20 min in the dark. Green fluorescence from JC-1 monomers (JC-1_{mon}) was recorded through FL1, while orange fluorescence from JC1-aggregates (JC-1_{agg}) was recorded through FL2 (cells with high mitochondrial membrane potential, HMMP).

2.6. Statistical analysis

The statistical analyses were conducted in the R statistical environment v. 4.3. Variables were first tested for normality and homogeneity of variances with Shapiro-Wilk test and Levene's test. Non-normal data were transformed with the Box-Cox prior to analysis. The effects of the treatment and storage were estimated using linear mixed-effects models. Treatment (CTR, P20 and P80) and the day of preservation (D0, D3 and D7) were used as fixed factors and the male as a random factor. Results are presented as mean ± SEM except if otherwise stated. The statistical significance was set at $P \leq 0.05$. Analysis of variance followed by the Dunnet and Bonferroni test was applied with *P* values as described by the figure captions.

3. Results

3.1. Cellular ATP production is affected by SLC and type of selection

The rate of cellular ATP synthesis, ATP production rate, was related to the simultaneous measurement of OCR in OxPhos (mitoATP production rate) and ECAR of the glycolytic pathway's conversion of glucose to lactate (glycoATP production rate). The results (Fig. 1) highlighted the relevance of a cellular metabolism based on substrate oxidation (mitochondrial predominance). Just after sample processing (day 0), we found an unaltered glycoATP production in all the conditions tested. In contrast, the mitoATP production increased by 75.8 % relative to the control in the sperm selected by P80 and was inhibited by 50.4 % in samples processed by P20. As a consequence, the Total ATP production of P80 was greater than the control, while that of P20 was less than the control. On day 3, the sperm energy profile of ATP production changed; P20 showed a reduction in ATP production, both aerobic and anaerobic, compared to the CTR, with the main production coming from the glycolytic pathway. On the other hand, the total ATP production of P80 was similar to the CTR, but its production was mainly aerobic, while the CTR showed predominantly glycoATP production. Hence, the ATP rate index of CTR and P20 was lower than 1, while P80 presented a value greater than 1. On day 7, there was an equalization of the total ATP production between P20 and P80, with a decrease in total ATP production of 69.3 % and 55.1 %, respectively, compared to the CTR (Fig. 1). In addition, an ATP index rate below 1 indicated inhibited mitochondrial activity, with anaerobic metabolism predominantly supported by glycolysis observed in all samples.

3.2. Energy map profiles of sperm cells

The phenogram condition of the metabolic state was evaluated, displaying the cell energy condition of quiescent (low level of ATP production), glycolytic (anaerobic ATP production), aerobic (mitochondrial OxPhos activity), and energetic (simultaneous activity of glycolysis and OxPhos). On day 0, CTR and, especially, P20 samples showed a quiescent profile, whereas P80 caused a change to the aerobic zone. On days 3 and 7, all treatments showed a drop into the quiescent quadrant (Fig. 2).

3.3. Flow cytometry results

3.3.1. Mitochondrial activity

Mitochondrial activity, measured in all cells using JC-1, decreased with colloid selection. This decrease intensified over time and

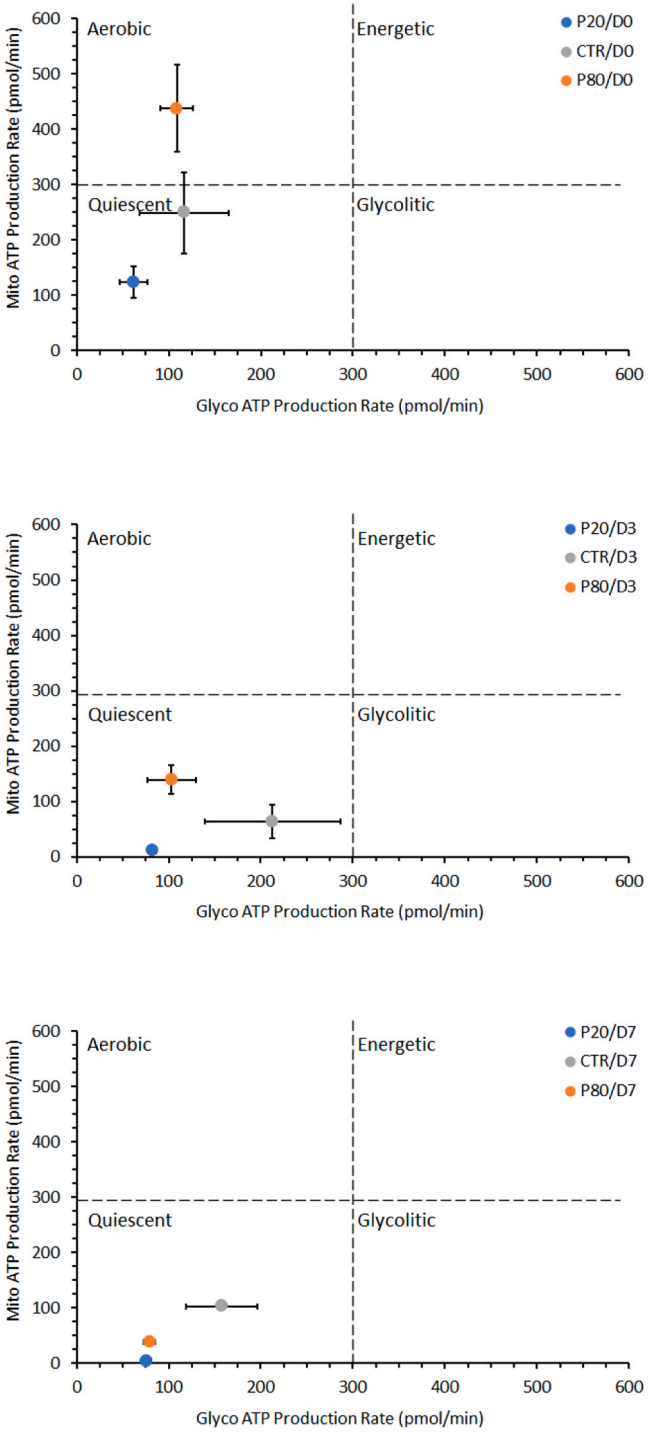

Fig. 2. Effect of time-dependent (D0, D3, and D7) treatments of CTR, P20, or P80 on the energy map relative to the metabolic state of sperm cells from boars (n = 3). Data, expressed as points, represent the mean ± SD (vertical and horizontal bars) from three experiments on distinct cell preparations.

was more pronounced in P80 (Fig. 3).

3.3.2. Cell viability

Colloid selection reduced sperm viability, as assessed by SYBR-14/PI, with the effect becoming more pronounced over time (Fig. 4).

3.3.3. Mitochondrial ROS production

Mitochondrial ROS production in live cells, measured by MitoSOX Red, did not significantly differ between treatments or over time (Fig. 5).

3.3.4. Motility

Table 1 shows the average results at days 0, 3, and 7 for sperm motility, including the p values from the linear mixed-effect models, with day and treatment as fixed factors. Sperm motility was overall higher in the CTR compared to the SLC treatments, with P80 causing a more pronounced decrease in motility, noticeable in some kinematic parameters even at D0 (Table 1). By D3, the effects of treatment and storage day effects were statistically significant for TM and PM. No differences were observed between the treatments in the other motility parameters evaluated. Nevertheless, P20 showed better maintenance of motility parameters on D7, with $p < 0.05$ for TM and VCL.

4. Discussion

Results obtained from the present research give some interesting insights into boar sperm metabolism during preservation and add some specific knowledge about the selection process achieved by SLC with Porcicoll® at different concentrations.

As we have already demonstrated, the oxidative metabolism of boar freshly ejaculated spermatozoa is characterized by a predominant mitochondrial activity, switching to a glycolytic one during refrigerated storage (Prieto et al., 2023). In other words, the ATP rate index switches from mitochondrial synthesis to glycolysis during storage to support ATP production, whereby two major metabolites for sperm metabolism are pyruvate and glucose (Rodríguez-Gil and Bonet, 2016). At different time points tested (D0, D3 and D7) the quantity of ATP synthesized from sperm cells underwent a 15 % decrease after three days due to a lower mitochondrial activity counterbalanced by an increase in the glycolytic pathway, as ATP production is higher through oxidative phosphorylation (OxPhos) than glycolysis (Du Plessis et al., 2015). Notably, the treatment with P80 at D0 permitted the selection of cells with the highest rate of ATP production, a less evident effect with the P20 treatment. This is consistent with the selecting capacity of the two different colloid concentrations. We can hypothesize that a colloid might be used to select sperm cells whose cell metabolism provided more energy by exploiting oxidative phosphorylation, with different selection grades due to colloid concentration.

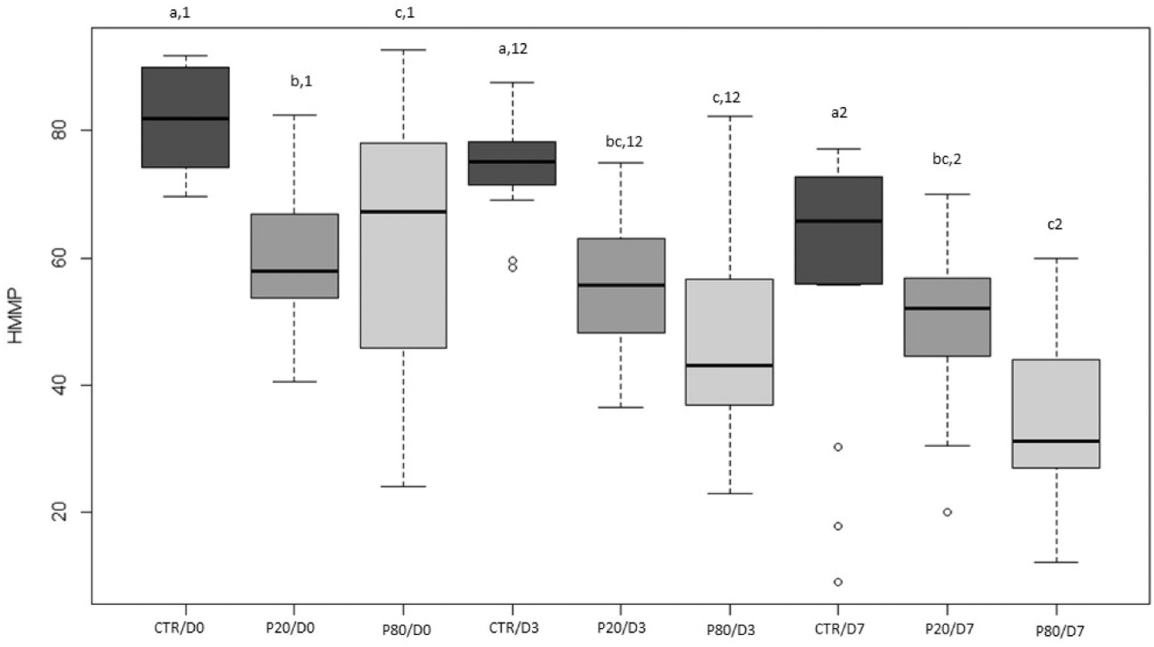

Fig. 3. Percentage of cells with high mitochondrial membrane potential (HMMP) in the different experimental groups into which boars (n = 15) were divided. Box plots represent the median (central line), 1st and 3rd quartiles (box), and maximum and minimum values (whiskers); dots outside the whiskers represent outliers. Comparisons were carried out using Dunnet's test with Bonferroni correction. Different letters indicate significant differences ($p < 0.05$) among treatments within the same experimental time point; different numbers indicate significant differences ($p < 0.05$) among time points within the same treatment.

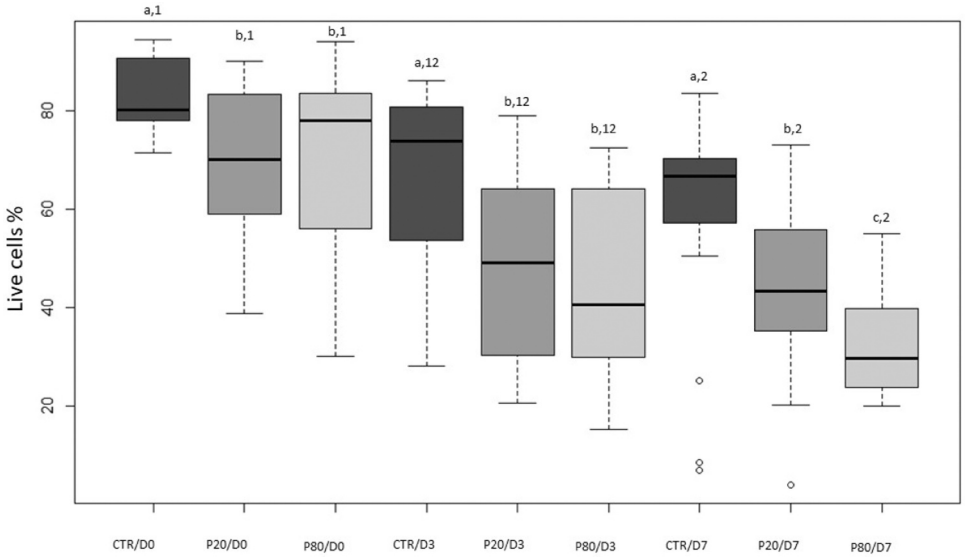

Fig. 4. Proportion of live cells in the different experimental groups into which boars (n = 15) were divided. Box plots represent the median (central line), 1st and 3rd quartiles (box), and maximum and minimum values (whiskers); dots outside the whiskers represent outliers. Comparisons were carried out using Dunnet's test with Bonferroni correction. Different superscripts letters indicate significant differences ($p < 0.05$) between treatments within the same experimental time point; different superscript numbers indicate significant difference ($p < 0.05$) between time points within the same treatment.

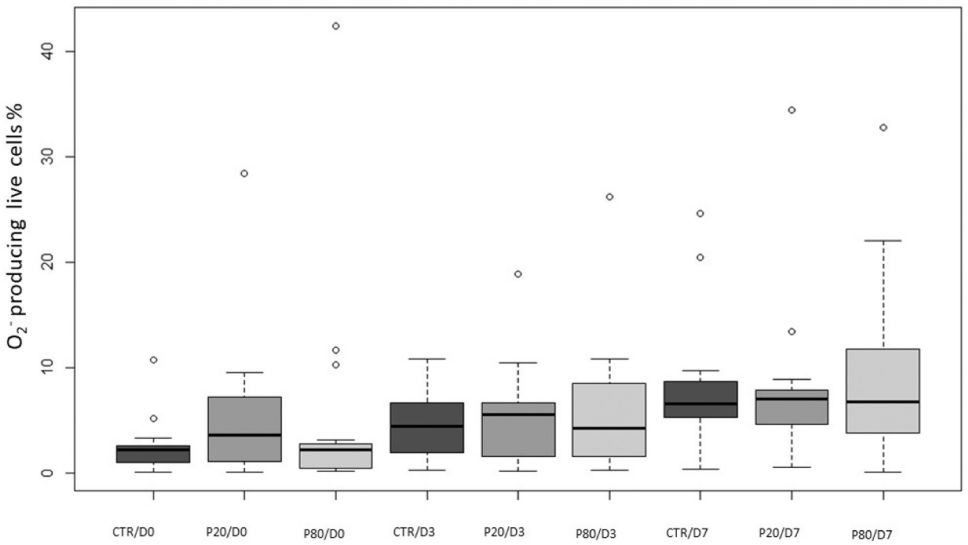

Fig. 5. Percentage of living cells producing mitochondrial O_2^- in the differently treated groups into which boars (n = 15) were divided. Box plots represent the median (central line), 1st and 3rd quartiles (box), and maximum and minimum values (whiskers); dots outside the whiskers represent outliers. Comparisons were conducted using Dunnet's test with Bonferroni correction (P > 0.05).

On the other hand, it might be pointed out that Androhep® is a medium useful for sperm conservation, including a high concentration of glucose (131.2 mM) without pyruvate (Taylor et al., 2009). Interestingly, after 3 and 7 days of liquid storage, all the samples switched their metabolism toward glycolysis. This is consistent with our previous observations in boar semen (Nesci et al., 2020; Prieto et al., 2023) and particularly evident in colloid-treated cells, precisely the 20 % colloid formulation. From our perspective, this is a significant result, as sperm cells selected on day 0 are the most metabolically active and those with more evident oxidative capacity. The aim of storing spermatozoa is to lower their metabolism by decreasing temperature (Waberski et al., 2019), which includes a switch toward glycolytic metabolism and a reduction of overall ATP production. These conditions enable the preservation of spermatozoa in the mid- to long-term, potentially reducing mitochondrial stress and preventing an increase in ROS production. Moreover, as indicated above, SLC allows for the complete removal of seminal plasma, whereas our CTR sample contains a small

Table 1
Descriptive statistics (median ± standard error) for the nine treatments into which boars (n = 15) were divided.

	TM	PM	VCL	VSL	VAP	LIN	STR	ALH	BCF
CTR/D0	$84.50 \pm 4.28^{a,1}$	$39.55 \pm 2.78^{a,1}$	$142.10 \pm 8.37^{ab,1}$	$53.50 \pm 2.77^{a,1}$	$75.80 \pm 3.84^{a,1}$	$39.70 \pm 2.18^{a,1}$	$69.60 \pm 3.38^{a,1}$	$5.66 \pm 0.37^{a,1}$	$39.90 \pm 1.99^{a,1}$
P20/D0	$81.20 \pm 4.29^{a,1}$	$40.62 \pm 2.79^{a,1}$	$177.20 \pm 8.37^{a,1}$	$71.30 \pm 2.77^{b,1}$	$96.00 \pm 3.84^{b,1}$	$40.30 \pm 2.18^{a,1}$	$70.30 \pm 3.38^{a,1}$	$6.84 \pm 0.37^{a,1}$	$38.30 \pm 1.99^{a,1}$
P80/D0	$72.20 \pm 4.29^{a,1}$	$48.35 \pm 2.79^{a,1}$	$137.20 \pm 8.37^{b,1}$	$69.30 \pm 2.77^{b,1}$	$81.70 \pm 3.84^{ab,1}$	$51.30 \pm 2.18^{b,1}$	$81.3 \pm 3.380^{a,1}$	$5.36 \pm 0.37^{a,1}$	$37.90 \pm 1.99^{a,1}$
CTR/D3	$71.70 \pm 4.34^{a,1}$	$13.80 \pm 2.87^{a,2}$	$161.80 \pm 8.39^{a,1}$	$36.70 \pm 2.80^{a,2}$	$71.10 \pm 3.85^{a,1}$	$24.40 \pm 2.18^{a,2}$	$52.30 \pm 3.38^{a,2}$	$6.71 \pm 0.37^{a,1}$	$34.70 \pm 1.99^{a,1}$
P20/D3	$50.40 \pm 4.34^{b,2}$	$6.54 \pm 2.87^{a,2}$	$158.30 \pm 8.39^{a,1}$	$34.10 \pm 2.80^{a,2}$	$69.60 \pm 3.85^{a,2}$	$23.00 \pm 2.18^{a,2}$	$48.30 \pm 3.38^{a,2}$	$6.55 \pm 0.37^{a,1}$	$36.40 \pm 1.99^{a,1}$
P80/D3	$30.70 \pm 4.34^{c,2}$	$5.94 \pm 2.87^{a,2}$	$141.00 \pm 8.39^{a,1}$	$29.50 \pm 2.80^{a,2}$	$58.40 \pm 3.85^{a,2}$	$21.90 \pm 2.18^{a,2}$	$48.40 \pm 3.38^{a,2}$	$5.47 \pm 0.37^{a,1}$	$37.20 \pm 1.99^{a,1}$
CTR/D7	$23.60 \pm 4.30^{ab,2}$	$3.04 \pm 2.80^{a,2}$	$99.40 \pm 8.38^{a,2}$	$25.80 \pm 2.78^{a,2}$	$45.60 \pm 3.84^{ab,2}$	$25.70 \pm 2.18^{a,2}$	$50.70 \pm 3.38^{a,2}$	$5.49 \pm 0.37^{a,1}$	$32.00 \pm 1.99^{a,1}$
P20/D7	$39.10 \pm 4.30^{a,2}$	$5.57 \pm 2.80^{a,2}$	$140.50 \pm 8.38^{b,1}$	$30.80 \pm 2.78^{a,3}$	$60.80 \pm 3.84^{a,2}$	$23.40 \pm 2.18^{a,2}$	$49.70 \pm 3.38^{a,2}$	$5.90 \pm 0.37^{a,1}$	$35.80 \pm 1.99^{a,1}$
P80/D7	$11.70 \pm 4.30^{b,3}$	$1.70 \pm 2.80^{a,2}$	$95.60 \pm 8.38^{a,2}$	$22.00 \pm 2.78^{a,2}$	$39.20 \pm 3.84^{b,2}$	$24.10 \pm 2.18^{a,2}$	$51.90 \pm 3.38^{a,2}$	$3.03 \pm 0.37^{b,2}$	$35.80 \pm 1.99^{a,1}$

Kinematics parameters of differently treated boar spermatozoa. TM – total motility; PM – progressive motility; VCL – curvilinear velocity (μm/sec); VAP – average path velocity (μm/sec); VSL -straight line velocity (μm/sec); LIN – linearity (%); STR straightness (%); BCF – beat cross frequency (Hz); ALH – amplitude of lateral head displacement (μm). Different superscript letters indicate a significant difference ($p < 0.05$) between treatments at the same day of evaluation and different superscript numbers indicate a significant difference ($p < 0.05$) between different time points within the same treatment.

amount (Bucci et al., 2013). A recent study has described that boar seminal plasma supplementation improves sperm quality by enhancing its antioxidant capacity during liquid conservation (Kou et al., 2022). This enhancement may help maintain a low ROS concentration, reduce oxidative stress, and preserve spermatozoa metabolism.

The results on ATP production were in line with the profile of the metabolic map. Indeed, the phenograms showed an aerobic profile with P80 at zero days; the time-dependent effect on energy metabolism reduced the ability of mitochondria to perform the ATP synthesis independently of the treatment. A relevant question is whether this situation could be reversed with a more prolonged exposure to physiological temperature (38 °C) after the storage since we analyzed the samples only a few minutes after returning to that temperature.

The other parameters that were studied aimed at defining sperm quality and mitochondrial activity. In general, we noticed decreased sperm quality after colloid centrifugation, which is not consistent with previous studies in different species (Blomqvist et al., 2011; Kruse et al., 2011; J. M. Morrell, 2006, 2019; J. M. Morrell, Dalin, et al., 2009; J. M. Morrell and Wallgren, 2011b, 2011a; van Wienen et al., 2011). In this regard, it should be noted that in previous work from our laboratories (Bucci et al., 2013) we registered membrane changes that might be considered early capacitation processes; this could explain the decrease in some functional parameters registered during preservation (viability, mitochondrial activity, and motility). Additionally, and in line with our observations on sperm metabolism modulation after SLC, the high glucose levels in Androhep® could negatively affect the selected spermatozoa. This hypothesis is based on findings about glucose toxicity during stallion sperm conservation (Ortiz-Rodriguez et al., 2021). SLC could increase the susceptibility of boar spermatozoa to the high concentration of glucose in the media, which would be adequate in unselected spermatozoa due to the lack of seminal plasma (and subsequent membrane changes, including adsorbed proteins) and to the mentioned metabolic modulation. Future studies might relate molecular events in boar sperm mitochondria during selection, refrigeration, and storage with the media composition. This information might help compare and improve storage protocols and extenders.

Also, the apparent discordance between bioenergetic study and the other sperm quality parameters could arise from the fact that we study metabolic profiles of living cells. At the same time, viability, mitochondrial activity, and motility are considered in the whole cell population. Finally, regarding ROS production, we focused on mitochondrial ROS production by the mean of a specific dye, MitoSOX™. Generally, we observed low ROS production, which tended to increase during preservation, irrespective of the different treatments. This trend is opposite to that observed for mitochondrial metabolism, where mitochondria are “shut down” while their ROS production was slightly (but not significantly) increased. This trend should be clarified, but it may be related to proton leak from the mitochondria during preservation or a decreased antioxidant capacity over time.

5. Conclusion

In conclusion, this study demonstrated that boar sperm metabolism changes due to preservation and is well maintained at 17 °C in non-selected spermatozoa with SLC. However, although SLC selects spermatozoa with higher ATP production, the complete elimination of seminal plasma and the media composition used in this study do not allow the preservation of sperm quality over time. Therefore, further studies on media composition and the fertility of selected sperm are required to enhance the application of sperm selection techniques in this species.

CRediT authorship contribution statement

Estibaliz Lacalle: Investigation, Writing - Original Draft. **Beatrice Mislei:** Data Curation, Resources **Cristina Algieri:** Formal analysis, Investigation. **Marcella Spinaci:** Methodology. **Felipe Martinez Pastor:** Validation, Formal analysis, Writing - Original Draft **Salvatore Nesci:** Writing – original draft, Visualization, Conceptualization, Supervision. **Jane M. Morrell:** Writing - Review & Editing. **Jose M. Ortiz-Rodriguez:** Writing - Review & Editing. **Diego Bucci:** Conceptualization, Visualization, Supervision, Writing - Original Draft

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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