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Selected Metabolites Found in Equine Oviductal Fluid do not Modify the Parameters Associated to Capacitation of the Frozen-thawed Equine Spermatozoa *In Vitro*

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ABSTRACT

In the horse, a repeatable protocol for *in vitro* fertilization has not been developed, possibly due to incomplete sperm capacitation. We have previously identified the metabolites present in equine oviductal fluid (OF). We aimed to test the effects of different metabolites found in equine oviductal fluid on quality parameters of frozen-thawed spermatozoa. Different concentrations of myoinositol (5–25 mM), lactate (6–60 mM), glycine (0.1–5 mM), β-alanine (1–6 mM), and histamine (0.05–0.4 mM) were added independently to modified Whitten's medium (pH = 7.25). Thawed equine spermatozoa (three stallions, one ejaculate per stallion, n = 3) were incubated for 2 hours at 37°C in presence of the selected metabolites. After sperm incubation, total motility (TM), and progressive motility (PM) were evaluated by computer-assisted sperm analysis. Viability (SYBR-14$^+$/PI$^-$), mitochondrial membrane potential ($\Delta\Psi$m) (JC-1), acrosome reaction (PNA$^+$/PI$^-$) and reactive oxygen species (ROS) production (CellRox$^+$/PI$^-$), were evaluated by flow cytometry. Protein tyrosine phosphorylation (PY) was evaluated by indirect immunofluorescence. Our results show that the addition of the metabolites at the dosages tested does not exert any effect on the sperm parameters analyzed. More research is needed to ascertain if metabolite addition at the dosages found in the equine OF exerts any remarkable effect on *in vitro* equine sperm capacitation.

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1. Introduction

Conventional *in vitro* fertilization (IVF) is an important assisted reproduction technique routinely used in domestic animals to produce transferrable embryos [5]. However, in horses there is still no repeatable conventional IVF protocol [24] being its low efficiency generally related to inefficient sperm capacitation. Sperm capacitation was described in 1951 by [3] and [8] and is a complex process that confers the spermatozoa the ability to fertilize an oocyte.

Several studies have been conducted in an attempt to determine the optimum environment required to induce *in vitro* capacitation in equine spermatozoa. In this regard, the fertilization medium has been supplemented with different substances and/or molecules such as bicarbonate and albumin [28], heparin [2], progesterone [35], leptin [22], alkaline pH [14], and also with equine oviducts explants cultured from epithelial cells [23]. Although some of these treatments result in increased protein tyrosine phosphorylation (PY) (a final event related to capacitation) or induced sperm hyperactivation and/or acrosome reaction, none of them have consistently shown to improve IVF success in horses.

In mammals, the oviductal environment plays a crucial role for gamete interaction and fertilization [29]; hence, different studies have been conducted to study its composition in order to design

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Ethical Statement: Ethical review and approval were not required for the animal study.

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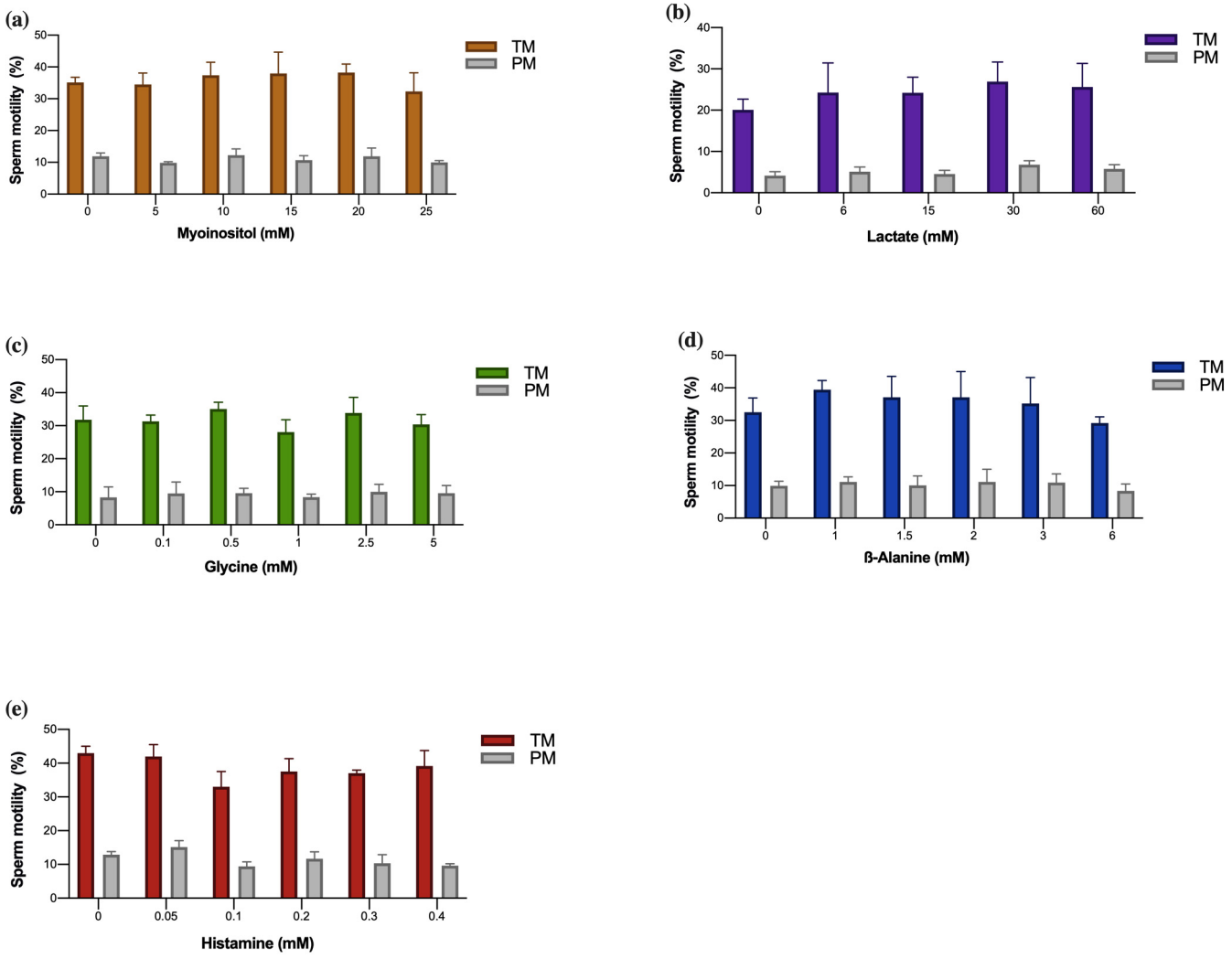

Fig. 1. Effect of different concentrations of myoinositol, lactate, glycine, β-alanine and histamine on sperm motility. Spermatozoa were incubated in MW medium with (A) myoinositol (5, 10, 15, 20, and 25 mM); (B) lactate (6, 15, 30, and 60 mM); (C) glycine (0.1, 0.5, 1, 2.5, and 5 mM); (D) β-alanine (1, 1.5, 2, 3, and 6 mM); and (E) histamine (0.05, 0.1, 0.2, 0.3, and 0.4 mM) for 2 hours at 37 °C in air. The bars represent the mean of the percentage of total motility (TM) and progressive motility (PM) ± SEM (n = 3).

an optimal *in vitro* fertilization medium. The oviductal fluid (OF) is secreted by oviductal epithelial cells and is a dynamic fluid composed of a mixture of different molecules such as ions, growth factors, proteins, hormones, and metabolites among others [32]. Many studies have tested the effect of OF in events related with capacitation in bull spermatozoa [20], boar spermatozoa [21] and ram spermatozoa [11], with the final objective of increasing *in vitro* fertilization rates.

Furthermore, the *in vitro* supplementation of specific metabolites found in the OF, have demonstrated to enhance fertilization or capacitation-related events in other species. As an example, the addition of myoinositol increases motility when added to frozen bovine spermatozoa [6] or fresh human spermatozoa [16]; glycine and ß-alanine are able to induce acrosome reaction in hamster spermatozoa [27] and pyruvate supplementation increases progressive motility, hyperactivation, and PY in human spermatozoa [18].

In this regard, we have previously reported that native OF increases PY in equine spermatozoa when added at very low dosages to a modified Whitten's medium [15]. As equine OF metabolomics has already been analyzed, based on our previous research we have selected candidate metabolites found in the oviductal fluid (myoinositol, lactate, glycine, ß-alanine, and histamine) and have added them individually at the concentrations found in OF to a classical capacitating medium. These metabolites were chosen based on their extracellular nature, the high concentration found in OF or their impact on capacitation-related events in spermatozoa of other species.

We aim to elucidate if any of the conditions tested improve equine sperm capacitation with the final goal of improving the composition of the classical fertilization media currently used in horses.

2. Material and methods

2.1. Chemicals and reagents

All reagents were purchased from Sigma-Aldrich Inc (Barcelona, Spain) unless otherwise stated.

2.2. Media

The basal incubation medium used was modified Whitten's (MW) medium [15]. MW was composed of 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (anhydrous), 22 mM HEPES,

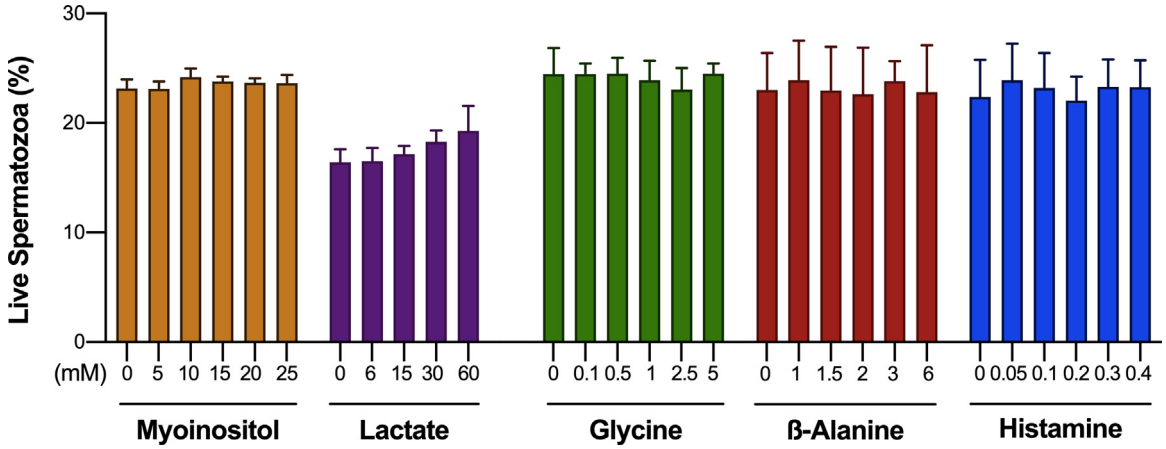

Fig. 2. Effect of different concentrations of myoinositol, lactate, glycine, β-alanine and histamine on sperm viability. Spermatozoa were incubated in MW medium with myoinositol (5, 10, 15, 20, and 25 mM), lactate (6, 15, 30, and 60 mM), glycine (0.1, 0.5, 1, 2.5, and 5 mM), β-alanine (1, 1.5, 2, 3, and 6 mM) and histamine (0.05, 0.1, 0.2, 0.3 and 0.4 mM) for 2 hours at 37 °C in air. The bars represent the mean of the percentage of live spermatozoa ± SEM (n = 3).

2.4 mM sodium lactate, 1.0 mM pyruvic acid, 25 mM bicarbonate, 2.4 mM calcium chloride, and 0.02% of polyvinyl alcohol (PVA) to prevent agglutination. A variant of MW (washing medium) was made omitting calcium chloride and bicarbonate; NaCl was added to adjust osmolarity. Media were adjusted to a pH of 7.25 prior sperm incubation.

2.3. Semen processing

Frozen semen was donated by the "Centro de Selección y Reproducción Animal of Extremadura" (CENSYRA), Spain. Ejaculates from three different stallions of proven fertility were used and each experiment was performed using one ejaculate from each of the three stallions (n = 3). Frozen semen was stored in liquid nitrogen in 0.5 mL French straws at 200 × 10^6 spermatozoa/mL. For each experiment, two straws were thawed in a water bath at 37°C for 1 minute. Afterwards, spermatozoa were centrifuged at room temperature (RT) in 1 mL of colloidal silica suspension (Pure Sperm 60%) for 10 minutes at 600 g. The pellet was washed in washing medium by centrifugation for 1 minute at 6,700 g at RT, and then diluted in MW medium at 15–20 × 10^6 spermatozoa/mL. MW was supplemented with different concentrations of myoinositol, lactate, glycine, β-alanine and histamine prior dilution and sperm suspensions were incubated at 37°C for 2 hours in a water bath. The control was added with NaCl to reach an equivalent osmolarity of the metabolite in study at the higher concentration used.

2.4. Evaluation of sperm motility

After incubation, sperm motility was analyzed using a computer-assisted sperm analysis (CASA) system (ISAS 1.0.6; Proiser S.L., Valencia, Spain). Two microliters of each sperm sample were placed in a warmed (37°C) counting chamber with a fixed height of 20 µm (Leja Standard Count two Chamber slides; Leja Products, B.V., Nieuw-Vennep, The Netherlands).

A minimum of four microscopic fields and at least 300 spermatozoa were evaluated for each sample. The parameters assessed were total motility (TM) and progressive motility (PM).

2.5. Flow cytometry

Flow cytometry analysis was performed using an ACEA NovoCyte flow cytometer (ACEA Biosciences, Inc, San Diego, CA, USA) equipped with a three detection channels for blue laser (488 nm): BL-1 (530 ± 30 nm band pass filter); BL-2 (572 ± 28 nm band pass filter) and BL-4 (675 ± 30 nm band pass filter) and a detection channel for a red laser (640 nm): RL-1 (675 ± 30 nm band pass filter). A total of 10,000 events at 400–800 cells/sec were acquired. Flow cytometry experiments and data analyses were performed using the ACEA Novo Express software (ACEA Biosciences, Inc, San Diego, CA, USA). Fluorescence data were acquired in a logarithmic scale.

After incubation, spermatozoa were evaluated by flow cytometry using different protocols described below. The samples were diluted to a final concentration of 1 × 10^6 spermatozoa/mL in phosphate buffered saline (PBS).

2.5.1. Analysis of sperm viability

Sperm viability was determined using SYBR-14 and Propidium Iodide (PI) probes (Thermo Fisher Scientific, Waltham, MA, USA) [12] at final concentration of 20 nM and 5 μM respectively. Samples were incubated for 10 minutes at RT. Viable spermatozoa were expressed as the percentage of SYBR-14 positive and PI negative ($SYBR\text{-}14^+$ and PI^-).

2.5.2. Analysis of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using the probe 5,5′,6,6′-tetrachloro-1,1′,3,3′- tetraethylbenzymidazolyl carbocianyne iodine (JC-1) [31] at a final concentration of 0.9 μM. Samples were incubated for 10 minutes at 37°C. The results were expressed in percentage of spermatozoa with high mitochondrial membrane potential (orange-stained cells).

2.5.3. Evaluation of acrosome reaction

The acrosome reaction was evaluated using the probes *Arachis hypogaea* lectin (PNA)-FITC and PI [31], at a final concentration of 0.4 μg/mL, and 1.2 μM respectively. Samples were incubated for 10 minutes at RT. Results were expressed as the average of the percentage of acrosome-reacted in live spermatozoa (PNA^+/PI^-).

2.5.4. Evaluation of reactive oxygen species production

The reactive oxygen species production was evaluated using the specific probe CellRox (Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 5 μM. Samples were incubated for 30 minutes at 37°C; PI was added at 4.8 μM in the last 5 minutes [30]. The fluorescence values were calculated on the geometric mean fluorescence intensity (MFI) of CellRox in live spermatozoa (PI^-). Results are expressed as the relative fluorescent intensity (RFI) in arbitrary units normalized to the control.

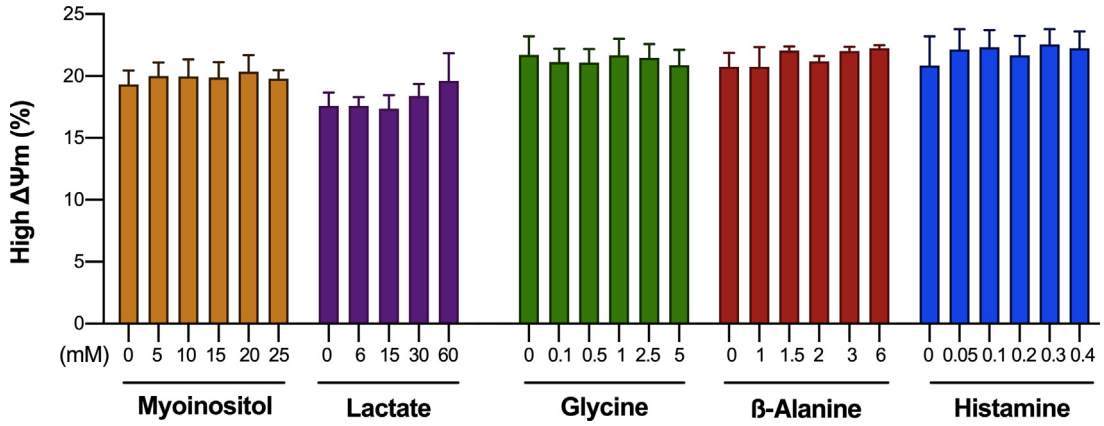

Fig. 3. Effect of different concentrations of myoinositol, lactate, glycine, β-alanine and histamine on mitochondrial membrane potential ($\Delta\Psi$m). Spermatozoa were incubated in MW medium with myoinositol (5, 10, 15, 20, and 25 mM), lactate (6, 15, 30, and 60 mM), glycine (0.1, 0.5, 1, 2.5, and 5 mM), β-alanine (1, 1.5, 2, 3, and 6 mM) and histamine (0.05, 0.1, 0.2, 0.3, and 0.4 mM) for 2 hours at 37 °C in air. The bars represent the mean of the percentage of spermatozoa with high mitochondrial membrane potential ± SEM (n = 3).

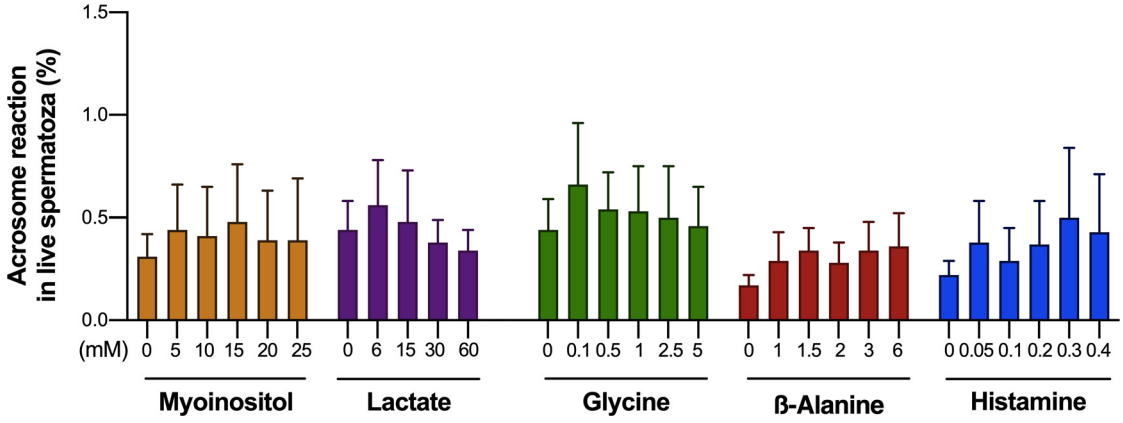

Fig. 4. Effect of different concentrations of myoinositol, lactate, glycine, β-alanine and histamine on acrosome reaction. Spermatozoa were incubated in MW medium with myoinositol (5, 10, 15, 20, and 25 mM), lactate (6, 15, 30, and 60 mM), glycine (0.1, 0.5, 1, 2.5, and 5 mM), β-alanine (1, 1.5, 2, 3, and 6 mM) and histamine (0.05, 0.1, 0.2, 0.3, and 0.4 mM) for 2 hours at 37 °C in air. The bars represent the mean of the percentage of acrosome-reacted in live spermatozoa ± SEM (n = 3).

2.5.5. Evaluation of protein tyrosine phosphorylation by indirect immunofluorescence

The protocol used was performed as previously described [13].

Following incubation, each sample was centrifuged for 3 minutes at 5,000 g. After centrifugation, the pellet was washed with 1 mL of PBS, and centrifuged again (3 minutes at 5,000 g). Then, spermatozoa were fixed with 4% formaldehyde in PBS for 15 minutes at RT, and washed with 1 mL of PBS for 1 minutes at 6,700 g. Spermatozoa were permeabilized with 0.1% Triton X-100 (v/v) in PBS for 10 minutes at RT. Spermatozoa were washed with PBS and blocked with 3% Bovine Serum Albumin (BSA) (w/v) in PBS for 60 minutes at RT. The samples were incubated with anti–phosphotyrosine monoclonal antibody 4G10 (diluted 1:500) in 3% BSA (w/v) in PBS at 4°C overnight.

The next morning, each sample was washed twice with 1% BSA in PBS (1 minute at 6,700 g), and then cells were incubated with goat anti–mouse IgG (H+L) Alexa FluorPLus 488 (2 mg/mL)-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) (diluted 1:500) in 3% BSA in PBS for 60 minutes at RT. After three washings with 1% BSA in PBS (1 minute at 6,700 g) each pellet was diluted in 25 μL of PBS and samples were mounted on a slide using ProLong gold antifade reagent with DAPI solution from Molecular Probes following manufacturer's indications (Eugene, OR, USA). Samples were then evaluated using an Olympus BX60 fluorescence microscope (New Hyde Park, NY, USA) equipped with a 60 x objective. A minimum of one hundred spermatozoa were counted for each sample. Spermatozoa were considered as positively stained when green fluorescence was detected along the tail.

2.6. Statistical analysis

The data were first examined using a Saphiro-Wilk test to confirm data distribution and analyzed for equal variances using a Leveneś test. A one-way ANOVA was used to compare values when normality was confirmed. A Kruskal-Wallis ANOVA on ranks test was used when the data did not show a gaussian distribution. Statistical significance was set at $P < .05$. Analyses were performed using SigmaPlot ver. 12.0 for Windows (Systat Software, Chicago, IL, USA).

3. Results

3.1. Evaluation of sperm motility and viability

First, we investigated the possible effects of the selected metabolites after sperm incubation on sperm motility, and viability. We did not observe statistically significant differences in any metabolite and concentration tested compared with its own control in total motility (TM: Fig. 1), progressive motility (PM; Fig. 1) or viability (Fig. 2).

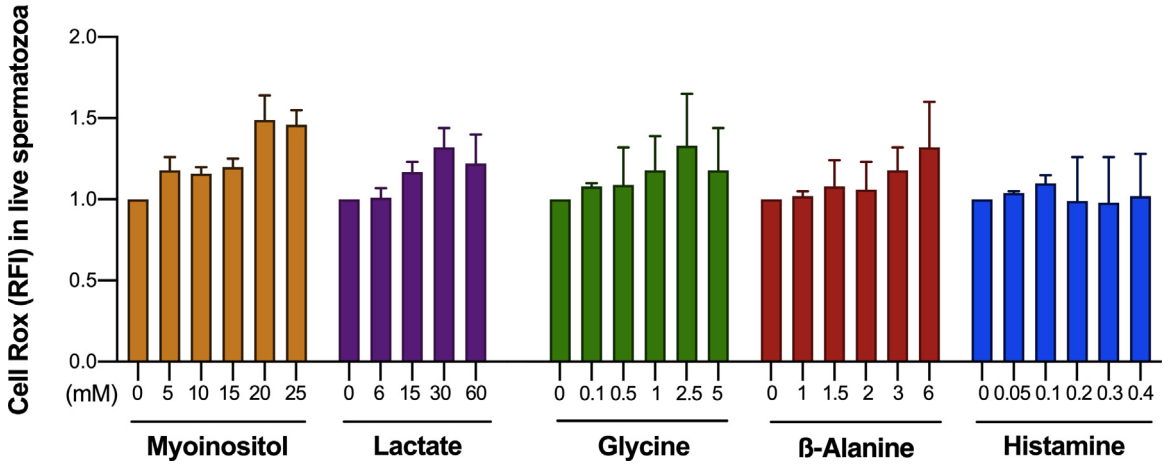

Fig. 5. Effect of different concentrations of myoinositol, lactate, glycine, β-alanine and histamine on reactive oxygen species production in live sperm. Spermatozoa were incubated in MW medium with myoinositol (5, 10, 15, 20, and 25 mM), lactate (6, 15, 30, and 60 mM), glycine (0.1, 0.5, 1, 2.5, and 5 mM), β-alanine (1, 1.5, 2, 3, and 6 mM) and histamine (0.05, 0.1, 0.2, 0.3, and 0.4 mM) for 2 hours at 37°C in air. The bars represent the relative fluorescent intensity (RFI) in arbitrary units of CellRox positive in live spermatozoa normalized to the control ± SEM (n = 3).

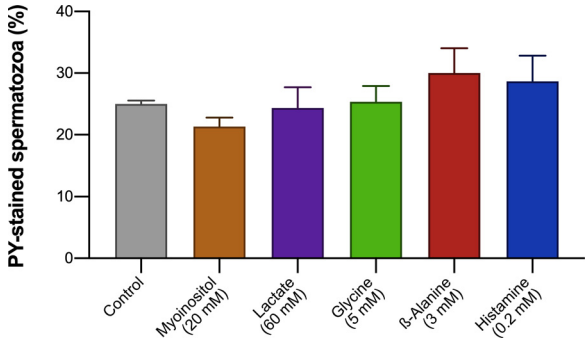

Fig. 6. Effect of different concentrations of myoinositol, lactate, glycine, β-alanine, and histamine on protein tyrosine phosphorylation. Spermatozoa were incubated in MW medium with 20 mM myoinositol, 60 mM lactate, 5 mM glycine, 3 mM β-alanine, and 0.2 mM histamine for 2 hours at 37°C in air. The bars represent the mean of the spermatozoa showing protein tyrosine phosphorylation along the entire tail ± SEM (n = 3).

3.2. Analysis of mitochondrial membrane potential, acrosome reaction, and reactive oxygen species production

Next, we investigated the effects of the selected metabolites on different sperm parameters. None of the sperm parameters studied were affected by any condition used when compared to its own control ($P > .05$): mitochondrial membrane potential (Fig. 3), acrosome reaction (Fig. 4), and reactive oxygen species production (Fig. 5).

3.3. Evaluation of protein tyrosine phosphorylation by indirect immunofluorescence

Finally, we studied the effect of the different metabolites at one of the concentrations found in the OF on PY. We did not observe statistically significant differences in the number of spermatozoa showing PY-associated fluorescence along the entire tail compared to the control (Fig. 6).

4. Discussion

In the present work, we aimed to assess the effect of specific metabolites found in the equine OF on equine frozen-thawed spermatozoa. To this end, we chose the candidate metabolites found in equine post-ovulatory OF [15] (myoinositol, lactate, glycine, β-alanine, and histamine).

The first metabolite analyzed was myoinositol, a polyalcohol that is mainly produced by Sertoli cells [9], and is known to be involved in sperm function regulation [7]. Myoinositol has been described to exert an antioxidant effect and play a role in the regulation of intracellular Ca^{+2} [4]. Interestingly, a study of the transcriptome of spermatozoa from fertile stallions showed a significantly enriched array of proteins related to D-myoinositol phosphate metabolism compared to subfertile stallions, suggesting that this metabolite may play a role in equine fertility [34]. However, in our conditions we did not observe any effect of myoinositol on sperm motility, mitochondrial membrane potential, acrosome reaction induction or changes in PY. These results are consistent with previous reports in which cooled equine sperm were supplemented with myoinositol [1] and no effects were observed.

In a previous work we demonstrated that the most concentrated metabolite found in equine OF prior and after ovulation is lactate [15]; this molecule is known to be a major source of energy for equine spermatozoa. It is well known that oxidative phosphorylation is the primary source of ATP that maintains motility and mitochondrial function in equine spermatozoa [10]; in this sense, it has been demonstrated that lactate, and pyruvate promote maximal mitochondrial function as equine sperm require a high rate of energy metabolism [10]. Even when typical capacitation media such as Modified Whitten's medium or Tyrode's albumin lactate pyruvate (TALP) contain lactate, it is added at concentrations ranging from 0.45 to 21.6 mM, which are significantly lower to what is found in equine oviductal fluid, whose concentration varies from 29.7 to 90 mM [15]. Hence, we hypothesized that a higher concentration of lactate could better mimic the oviductal environment, and enhance equine spermatozoa ability to undergo capacitation. However, in our setting, lactate addition did not induce any significant change in equine sperm motility, acrosome reaction or PY induction after 2 hours. These results are in contrast to those reported by Hernández-Avilés, *et al.* who described that lactate addition at 19.8, 40- or 80-mM decreased sperm motility and acrosome intactness after 1 hour of incubation at 37°C [19]. However, these authors used a skimmed-milk based extender added with 10% seminal plasma to perform their incubations and the osmolarity of the medium was adjusted using sucrose. Hence, these divergences in the experimental design may explain why in our setting no differences in the sperm parameters assessed were detected.

In our work, the effects of two additional metabolites namely glycine, and ß-alanine were tested. Both metabolites trigger the activation of the glycine receptor/Cl- (GlyR) that induces acrosome reaction [27] as demonstrated in human, porcine, mouse, and hamster spermatozoa [33]. In our experimental setting, the induction of acrosome reaction was not observed at any of the dosages tested for glycine or ß-alanine. These differences with previous reports can be attributed to the fact that our spermatozoa were incubated in a capacitating medium in presence of glycine or ß-alanine, while in previous reports in hamster spermatozoa, 3 hours of precapacitation prior amino acid addition were used [27]; besides, the zona pellucida seems to be required to properly trigger GlyR activation, and in our setting, no oocytes were present [25].

The last metabolite tested was histamine, which has been reported to trigger an increase in intracellular calcium levels in human spermatozoa that lead to cell death in dosages ranging from 50 to 165 mM [17], well above the 0.08–0.37 mM histamine concentration found in equine OF [15]. In our setting, no deleterious effects were observed when histamine was added at any dosage tested, and no effect on capacitation related events was noticed either. Hence, as previously postulated, histamine may be involved in the regulation of smooth muscle contractions in the oviduct, promoting sperm progression [26].

Unfortunately, none of the metabolites tested in the present work at the dosages found in the equine oviduct seemed to promote sperm capacitation. However, no deleterious effects on sperm viability were observed, and hence, the dosages tested demonstrated to be non–toxic. Our data failed to show a single metabolite and/or dosage that enhanced capacitation-related events *in vitro*, but these metabolites may need to be tested in combination. The use of frozen spermatozoa in our experimental setting tried to mimic the most common scenario found in IVF laboratories in which frozen semen is generally used, however, the use of fresh equine spermatozoa could also yield different results, and more studies should be conducted.

5. Conclusion

Our data are the first to analyze the effects of selected metabolites at the concentrations found in equine oviductal fluid on equine spermatozoa. Even when our results did not show any significant change in any of the parameters analyzed, we keep increasing our understanding of the equine sperm capacitation requirements aiming to develop a repeatable equine *in vitro* fertilization protocol. More research is warranted to better understand the capacitation requirements of equine spermatozoa and the role of the oviductal environment during *in vitro* fertilization.

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