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Participation of phosphofructokinase, malate dehydrogenase and isocitrate dehydrogenase in capacitation and acrosome reaction of boar spermatozoa

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Contents

The aim of this work was to determine the enzymatic activity of phosphofructokinase (PFK), malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH) in boar spermatozoa and study their participation in bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction. Enzymatic activity of these enzymes was determined spectrophotometrically in extracts of boar spermatozoa. Sperm suspensions were incubated in the presence of bicarbonate (40 mM), a well-known capacitation inducer, or follicular fluid (30%), as an acrosome reaction inducer, and different concentrations of oxoglutarate, oxalomalate and hydroxymalonate, inhibitors of PFK, IDH and MDH, respectively. Capacitation percentages were determined by the fluorescence technique of chlortetracycline (CTC), and true acrosome reaction was determined by trypan blue and differential–interferential contrast, optical microscopy. The activity of PFK in boar spermatozoa enzymatic extracts was 1.70 ± 0.19 U/10^{10} spermatozoa, the activity of NAD- and NADP-dependent IDH was 0.111 ± 0.005 U/10^{10} and 2.22 ± 0.14 U/10^{10} spermatozoa, respectively, and the activity of MDH was 4.24 ± 0.38 U/10^{10} spermatozoa. The addition of the specific inhibitors of these enzymes prevented sperm capacitation and decreased sperm motility during capacitation and inhibited the acrosome reaction (AR), without affecting the sperm motility during this process. Our results demonstrate the participation of PFK, IDH and MDH in bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction in boar spermatozoa, contributing to elucidate the mechanisms that produce energy necessary for these processes in porcine spermatozoa.

1 | INTRODUCTION

Mammalian spermatozoa require a preparation period denominated capacitation to acquire the ability to fertilize mature oocytes. During this process, changes in plasma membrane fluidity, O_2 uptake, metabolism, intracellular ionic concentration and the activity of several enzymes take place. Capacitation is followed by an exocytotic event termed acrosome reaction that is an absolute requisite for fertilization (Yanagimachi, 1994). In boar spermatozoa, capacitation is dependent upon calcium and bicarbonate, but does not appear to require albumin, possibly due to the remarkably low cholesterol/phospholipid ratio of boar spermatozoon (Althouse, 1992; Tardif, Dubé, & Bailey, 2003). Bicarbonate has been identified as the essential capacitating agent for several animal species, including the pig (Harrison & Gadella, 2005). It has been shown that bicarbonate induces rapid and major changes in plasma membrane architecture (Harrison, 1996) and increases motility (Holt & Harrison, 2002); these two events are both mediated through bicarbonate-induced stimulation of soluble adenylyl cyclase activity (Litvin, Kamenetsky, Zarifyan, Buck, & Levin, 2003).

Acrosome reaction (AR) involves fusion between the outer acrosomal membrane and the overlying spermatozoa plasma membrane, and exposure of acrosomal enzymatic contents (Yanagimachi, 1994). Sperm capacitation has been confirmed by measuring the ability of spermatozoa to respond to human follicular fluid by undergoing acrosome reaction (Herrero, de Lamirande, & Gagnon, 2001).

Spermatozoa are dependent on an efficient generation of ATP to fuel progressive motility, capacitation, hyperactive motility and AR crucial for fertilization (Ho, Granish, & Suarez, 2002). Boar spermatozoa have a high affinity for hexoses for being used in the glycolytic pathway for the production of ATP as well as for non-hexose compounds such as the glycolytic end products pyruvate and lactate (Jones, 1997). Despite the large controversy on this topic, several authors concluded that the most important metabolic pathway by which mature boar spermatozoa obtain energy is glycolysis (Rodríguez-Gil, 2013). In a metabolomic study, it has been observed that approximately 95% of the energy yielded by glucose in boar spermatozoa is originated by the glycolytic pathway (Marin et al., 2003). The environment that mature boar spermatozoa will find after ejaculation inside the female genital tract would be mostly anaerobic. On the other hand, it has also been observed that the achievement of a feasible, progesterone-induced in vitro AR is concomitant with a rapid and intense increase in the oxygen consumption rate, which indicates a rapid, transitory and intense peak of the mitochondrial respiratory activity (Ramio-Lluch et al., 2011). This seems to indicate that this minority energy would be necessary for the achievement of acrosome reaction; meanwhile, glycolysis-synthesized energy would be the main energy source for all other boar spermatozoa necessities (Rodríguez-Gil, 2013). However, information regarding the mechanisms to control energy support in boar spermatozoa is still scarce.

The third step of glycolysis is catalysed by phosphofructokinase (PFK), which is the most important rate-limiting enzyme of glycolysis. This enzyme catalyses the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate; it is an allosteric enzyme comprised of four subunits and controlled by several positive and negative effectors (Nelson & Cox, 2004). The tricarboxylic acid cycle (Krebs cycle) is a complex pathway involving several enzymes and metabolic intermediates. The components of this cycle and their modulation through changes in cell physiology have been well appreciated for over 60 years (Garrett & Grisham, 2007; Krebs, 1940). Isocitrate dehydrogenase (IDH) catalyses oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD^+ or $NADP^+$ (Kil & Park, 2005). IDH enzymatic activity is present in three different forms, two mitochondrial [NAD(P)] and one cytosolic (NADP) (Clarenburg, 1992). The NAD^+-dependent isoform is the most important regulatory step of the Krebs cycle. Malate dehydrogenase (MDH) catalyses the NAD/NADH-dependent interconversion of the substrates malate and oxaloacetate. This reaction plays a key part in the malate/aspartate shuttle across the mitochondrial membrane, and in the tricarboxylic acid cycle within the mitochondrial matrix (Minarik, Tomaskova, Kollarova, & Antalik, 2002).

In a thorough study, Travis et al. (2001) have dismissed hexokinase as a major site of glycolytic control in murine spermatozoa, while Jones & Connor (Jones & Connor, 2004) have provided evidence that control must be located between hexokinase and aldolase in boar spermatozoa. These results point to PFK as a likely candidate because in many tissues and cell types, this enzyme has been identified as the main regulatory step of glycolysis. Moreover, in boar spermatozoa, PFK has been localized in the mid-piece and principal piece of the flagellum, as well as in the acrosomal area at the top of the head (Kamp et al., 2007).

The 2D gel analysis revealed that sperm cells incubated under capacitating conditions showed increased expression levels of MDH, IDH, vacuolar ATP synthase catalytic subunit α and pyruvate dehydrogenase compared with control, non-capacitated sperm cells (Choi et al., 2008). The levels of MDH increased significantly during capacitation and spontaneous acrosome reaction, indicating that participation of the malate–aspartate shuttle may be required to maintain the levels of reduced coenzyme necessary for capacitation and spontaneous AR (Choi et al., 2008). Proteomics studies by different laboratories also confirmed the presence of IDH in human spermatozoa (Amaral, Castillo, Ramalho-Santos, & Oliva, 2014; Baker et al., 2007; Wang et al., 2013); whether the IDH is involved in human sperm capacitation remains unknown.

Taking into account that PFK, IDH and MDH are enzymes that play a pivotal role in energy generation pathways and that capacitation and AR are complex processes that require energy, the aim of this work was to study the participation of PFK, IDH and MDH in boar sperm capacitation and acrosome reaction by determining their enzymatic activity and the effect of their inhibition on these processes.

2 | MATERIALS AND METHODS

2.1 | Materials

Dextrose, sodium citrate, sodium bicarbonate, sodium chloride, EDTA, potassium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate were from Merck (Darmstadt, Germany). Trypan blue, eosin yellow and nigrosine were purchased from Mallinckrodt (St. Louis, MO, USA). Glucose, sodium pyruvate, bovine serum albumin (BSA), 2-oxoglutarate, oxalomalate and hydroxymalonate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 | Semen collection

Samples were collected by the gloved-hand technique (Hancock & Hovell, 1959; Pursel & Johnson, 1975) from four cross-bred boars (Pietrain x Yorkshire) of proven fertility, 1–1.5 year of age. These boars belonged to a controlled reproduction programme and were kept under uniform feeding and handling conditions during the entire study. All animal experiments were performed in accordance with the guidelines of the Institutional Committee for Care and Use of Experimental Animals of the *Facultad de Ciencias Veterinarias*, *Universidad de Buenos Aires*. Only samples with a minimum of 70% motile and 80% viable spermatozoa (evaluated by the viability and pattern F of CTC parameters) were included in the study.

2.3 | Preparation of enzymatic extracts

Fresh semen samples were diluted (1:1) in BTS buffer [3.7 g/100 ml dextrose, 0.6 g/100 ml sodium citrate, 0.125 g/100 ml $NaHCO_3$, 0.125 g/100 ml EDTA, 0.075 g/100 ml KCl], and after 10 min of incubation at 37°C, samples were centrifuged at 600 *g* to separate seminal plasma. Spermatozoa were suspended in distilled water, and the suspension was frozen at −20°C until use (maximum 2 months). The frozen sperm suspensions were thawed, homogenized and sonicated at 100 W in 50% cycle at 4°C using a Vibra-Cell sonicator model 600W (Sonics & Materials Inc, Newton, USA) for 4 min. After centrifugation of the homogenate (17,000 *g*, 20 min, 4°C), the supernatants were collected and maintained at 4°C until enzyme assays. For all the enzymes, the extracts were prepared to obtain a final amount of enzyme that ensures linear behaviour during the time of activity measurement, thus enabling to calculate the rate of absorbance variation per minute.

2.4 | Preparation of the sperm suspension for capacitation

Fresh semen was diluted (1:1 [v/v], semen: PBS-BSA 0.1%), held in a water bath at 38°C for 10 min and centrifuged at 600 *g* for 5 min. Spermatozoa were suspended in capacitating medium [modified TRIS-buffered medium, mTBM: 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 20 mM TRIS, 11 mM glucose, and 5 mM sodium pyruvate], to a final concentration of 3×10^7 spermatozoa/ml for all the experiments. Sperm suspensions were incubated for 120 min at 38°C under 5% CO_2 in humidified air in the presence or absence of $NaHCO_3$ (40 mM) as a capacitation inducer (Satorre, Breininger, Beconi, & Beorlegui, 2007). Capacitating medium was monitored for pH (7.4) and osmolarity (340 mOsm).

2.5 | Preparation of the sperm suspension for acrosome reaction (AR)

Samples of previously capacitated spermatozoa corresponding to each treatment were incubated for 15 min at 38°C under 5% CO_2 in humidified air in the presence of 30% follicular fluid as an AR inducer (Breininger, Beorlegui, & Beconi, 2005).

2.6 | Determination of phosphofructokinase activity

PFK activity in spermatozoa extracts was measured in a Shimadzu spectrophotometer model UV-160 (Shimadzu Corporation, Tokyo, Japan) at 340 nm for 10 min at 37°C in a TRIS–HCl buffer (100 mM, pH 8.2) supplemented with (assay concentrations) 10 mM $MgCl_2$, 10 mM NH_4Cl, 4 mM fructose 6-phosphate, 0.4 mM NADH, 2 mM ATP and 1 mM AMP, and the following auxiliary enzymes: 1.4 U aldolase, 40 U phosphotriose isomerase and 5 U glycerol-3-phosphate dehydrogenase (Kotlarz & Buc, 1982). An enzymatic unit of PFK was defined as the quantity of enzyme that catalysed the production of 1 μmol of fructose 1,6-bisphosphate per minute that was equal to the oxidation of 2 μmol of NADH per minute.

2.7 | Determination of NAD-dependent isocitrate dehydrogenase activity

NAD-dependent IDH activity in spermatozoa extracts was measured in a Shimadzu spectrophotometer model UV-160 (Shimadzu Corporation, Tokyo, Japan) at 340 nm for 10 min at 37°C in a TRIS–HCl buffer (70 mM, pH 7.1) supplemented with (assay concentrations) 8 mM $MgCl_2$, 1 mM $MnCl_2$, 1.5 mM isocitrate, 10 mM citrate, 2 mM ADP and 2 mM NAD (Alp, Newsholme, & Zammit, 1976). An enzymatic unit of NAD-dependent IDH was defined as the quantity of enzyme that catalysed the reduction of 1 μmol of NAD per minute.

2.8 | Determination of NADP-dependent isocitrate dehydrogenase activity

NADP-dependent IDH activity in spermatozoa extracts was measured in a Shimadzu spectrophotometer model UV-160 (Shimadzu Corporation, Tokyo, Japan) at 340 nm for 10 min at 37°C in a TRIS–HCl buffer (70 mM, pH 7.5) supplemented with (assay concentrations) 8 mM $MgCl_2$, 1 mM $MnCl_2$, 1.5 mM isocitrate, 10 mM citrate and 0.5 mM NADP (Alp et al., 1976). An enzymatic unit of NADP-dependent IDH was defined as the quantity of enzyme that catalysed the reduction of 1 μmol of NADP per minute.

2.9 | Determination of malate dehydrogenase activity

MDH activity in spermatozoa extracts was measured in a Shimadzu spectrophotometer model UV-160 (Shimadzu Corporation, Tokyo, Japan) at 340 nm for 1.5 min at 37°C in a glycine buffer (90 mM, pH 10) supplemented with (assay concentrations) 40 mM malate and 0.5 mM NAD (Kitto, 1969). An enzymatic unit of MDH was defined as the quantity of enzyme that catalysed the reduction of 1 μmol of NAD per minute.

2.10 | Determination of total proteins

Total protein concentration was determined in enzymatic extract supernatants using the method described by Lowry, Rosebrough, Farr, & Randall (1951). Briefly, 10 μl of enzymatic extract was added into biuret reagent and further incubated for 10 min at room temperature. Thereafter, 100 μl Folin–Ciocalteu reagent was added into the reaction mixture and further incubated at room temperature in darkness for other 30 min. The calibration curve was made using different dilutions of a solution of BSA (1 mg/ml). All determinations were made at 660 nm.

2.11 | Evaluation of motility and sperm viability

Total sperm motility was evaluated three times by the same observer using an optical microscope equipped with a thermal stage at 37°C at 400× magnification (Binocular Microscope XSZ 100 BN, Arcano, China). The percentage of live spermatozoa was determined by the

supravital eosin/nigrosine technique (Satorre et al., 2007). Staining was carried out by mixing an aliquot of sperm suspension of each treatment with the eosin–nigrosine solution (1:3 dilution) for 30 s before preparing a smear and drying on a warm plate at 37°C. At least 200 spermatozoa were counted in each sample at an optical microscope (Binocular Microscope XSZ 100 BN, Arcano, China) at 400× magnification.

2.12 | Determination of sperm concentration

Sperm concentration was determined by haemocytometry using a Neubauer chamber. An average of the count of both chambers was used for each determination.

2.13 | Determination of sperm capacitation

Sperm capacitation status was evaluated through the modifications in fluorescence of CTC patterns as described by Wang, Abeydeera, Fraser, & Niwa (1995). Three patterns were observed: F (fluorescent), intact non-capacitated spermatozoa displaying fluorescence throughout their surface; C (capacitated), intact capacitated spermatozoa that lost fluorescence in the post-acrosomal region; AR (acrosome-reacted), spermatozoa with a reacted acrosome that lost fluorescence in the post-acrosomal and acrosomal regions, exhibiting fluorescence only in the mid-piece.

CTC solution (500 μM CTC, 130 mM NaCl, 5 mM DL-cysteine, 20 mM TRIS–HCl, pH 7.8) was mixed with an equal volume of the medium containing the capacitated spermatozoa (3×10^7 spermatozoa/ml). Glutaraldehyde was then added to the mixture reaching a final concentration of 0.1%. Slides were examined at 400× magnification under epifluorescence excitation at 410 nm using a Carl Zeiss Jena Jenamed 2 epifluorescence microscope.

2.14 | Determination of true acrosome reaction

An aliquot of the sperm suspension of the different treatments was incubated with the same volume of trypan blue solution (0.25% m/v), during 15 min at 38°C. To remove the excess of stain, it was centrifuged 10 min at 600 *g* and suspended in PBS with 5% of formaldehyde. Reacted acrosomes were evaluated in live and dead spermatozoa by differential–interferential contrast (DIC), optical microscopy (Garde, Ortiz, Garcia, & Gallego, 1997; O'Flaherty, Beorlegui, & Beconi, 1999).

2.15 | Participation of PFK, IDH and MDH in boar sperm capacitation

To discern the possible participation of PFK, IDH and MDH in boar sperm capacitation, sperm suspensions (n = 5) were incubated, as described earlier, in mTBM supplemented with bicarbonate and different concentrations of oxoglutarate (specific inhibitor of the PFK), oxalomalate (specific inhibitor of the IDH) or hydroxymalonate (specific inhibitor of the MDH; Chang, Scheer, Grote, Schomburg, & Schomburg, 2009). Stock solutions of the inhibitors were prepared in mTBM, to which pH and osmolarity were carefully adjusted to 7.4 and 340 mOsm if necessary. CTC patterns, total sperm motility and sperm viability were evaluated in each treatment.

2.16 | Acrosome reaction induced by follicular fluid in spermatozoa capacitated with bicarbonate in the presence of specific enzyme inhibitors

To confirm the participation of PFK, IDH and MDH in boar sperm capacitation, the acrosome reaction (AR) was induced for 15 min by the addition of 30% follicular fluid (Breininger et al., 2005) to sperm suspensions (n = 5) previously capacitated in the presence of the 20 mM oxoglutarate, 10 mM oxalomalate and 0.5 mM hydroxymalonate. Acrosome reaction, total sperm motility and viability were evaluated.

2.17 | Participation of PFK, IDH and MDH in boar sperm AR

To discern the possible participation of PFK, IDH and MDH in boar sperm AR, suspensions of previously capacitated spermatozoa (n = 5) were incubated for 15 min at 38°C, in the presence of 30% follicular fluid and different concentrations of oxoglutarate, oxalomalate or hydroxymalonate. Acrosome reaction, total sperm motility and sperm viability were evaluated in each treatment.

2.18 | Statistical analysis

Percentages of total sperm motility, sperm viability, capacitated sperm, and acrosome-reacted live spermatozoa and enzymatic activities (expressed as enzymatic units per 10^{10} spermatozoa and enzymatic units per mg protein) are given as means ± *SEM*. Two ejaculates of four boars and five replicates per sample were analysed. For the analysis of the effect of the treatments in the different experiments, an analysis of variances was performed (ANOVA) and the Bonferroni test was used as a post-ANOVA. Analysis of covariance was previously performed to ensure that the differences in semen quality among samples did not interfere in the results obtained. A value of $p < 0.05$ was considered as statistically significant. All statistical tests were performed using the software InfoStat (*Universidad de Córdoba, Córdoba, Argentina*, see http://www.infostat.com.ar/).

3 | RESULTS

Enzymatic activities of PFK, IDH and MDH were determined in the absence or presence of their specific inhibitors in boar spermatozoa. To standardize the results against the protein content of the spermatozoa, we determine the activity in enzyme units (U/10^{10} spermatozoa) and specific activity (U/mg protein). The activity of MDH was approximately the double of the PFK activity for both enzyme units and enzymatic activity, being the NAD-dependent IDH enzymatic activity the lower (Table 1).

TABLE 1 PFK, IDH and MDH enzymatic activities in porcine spermatozoa

	PFK	IDH-NAD	IDH-NADP	MDH
Units per 10^{10} spermatozoa	1.700 ± 0.200[a]	0.111 ± 0.005[b]	2.226 ± 0.143[a]	4.244 ± 0.385[c]
Units per mg protein	0.0075 ± 0.0004[a]	0.00029 ± 0.0002[b]	0.0097 ± 0.0073[a]	0.0228 ± 0.0032[c]

Enzymatic activities are expressed in units per 10^{10} spermatozoa (U) and units per mg protein. Values are means ± *SEM* of five replicates. For each parameter, the superscripts a-c indicate significant differences ($p < .05$).

TABLE 2 Inhibition of PFK, IDH and MDH enzyme activity in porcine spermatozoa

Oxoglutarate	0 mM	20 mM	30 mM
U of PFK	1.700 ± 0.200[a]	1.020 ± 0,150[b]	0.080 ± 0.020[b]
% inhibition	–	41 ± 7	94 ± 2
Oxalomalate	0 mM	10 mM	20 mM
U of IDH-NAD	0.111 ± 0.005[a]	0.047 ± 0. 006[b]	0.021 ± 0.002[c]
% inhibition	–	57 ± 4	81 ± 4
Oxalomalate	0 mM	10 mM	20 mM
U of IDH-NADP	2.226 ± 0.143[a]	1.100 ± 0.260[b]	0.607 ± 0.082[c]
% inhibition	–	51 ± 3	73 ± 2
Hydroxymalonate	0 mM	0.5 mM	5 mM
U of MDH	4.244 ± 0.385[a]	1.730 ± 0.182[b]	0.971 ± 0.202[c]
% inhibition	–	58 ± 8	75 ± 5

Enzymatic activities are expressed in units per 10^{10} spermatozoa (U). Values are means ± *SEM* of five replicates. For each enzyme, the superscripts a-c indicate significant differences ($p < .05$) in presence or absence of specific inhibitors.

The activity of PFK in spermatozoa enzymatic extracts was almost completely inhibited (94 ± 2%) by the addition of 30 mM of oxoglutarate. The enzymatic IDH-NAD and IDH-NADP was inhibited (81 ± 4% and 73 ± 2%, respectively) by the addition of 20 mM of oxalomalate. The activity of MDH was mostly inhibited (75 ± 5%) by the addition of 5 mM of hydroxymalonate (Table 2).

The specific inhibitor of the PFK, oxoglutarate, was used to confirm the participation of glycolytic pathway in bicarbonate-induced capacitation of boar spermatozoa. Capacitation was entirely blocked by 30 mM oxoglutarate (Figure 1a), and a dose-dependent decrease ($p < .05$) in sperm motility during capacitation was also observed. Sperm viability was not affected by oxoglutarate at any of the concentrations used (Figure 1b).

Oxalomalate, specific inhibitor of the IDH, was used to confirm the participation of this enzyme in bicarbonate-induced capacitation of boar spermatozoa. Capacitation was inhibited completely by 20 mM oxalomalate (Figure 2a), and a dose-dependent decrease ($p < .05$) in sperm motility during capacitation was also observed. Sperm viability was not affected by oxalomalate at any of the concentrations used (Figure 2b).

The specific inhibitor of the MDH, hydroxymalonate, was used to confirm the participation of this enzyme in bicarbonate-induced capacitation of boar spermatozoa. Capacitation was entirely blocked by 5 mM hydroxymalonate (Figure 3a), and a dose-dependent decrease ($p < .05$) in sperm motility during capacitation was also observed. Sperm viability was not affected by hydroxymalonate at any of the concentrations used (Figure 3b).

To confirm the inhibition of sperm capacitation by these inhibitors, the AR was induced by the addition of 30% follicular fluid to capacitated boar spermatozoa in the presence of the 20 mM oxoglutarate, 10 mM oxalomalate and 5 mM hydroxymalonate. AR was significantly diminished ($p < .05$) in all these treatments, without affecting motility or sperm viability (Table 3).

AR was induced by the addition of 30% follicular fluid to previously capacitated boar spermatozoa. A significant dose-dependent decrease was observed in response to the addition, during follicular fluid-induced AR, of specific inhibitors of PFK, IDH and MDH (Figure 4). With 10 mM of oxoglutarate (PFK-specific inhibitor), the AR was significantly diminished ($p < .05$, Figure 4a) without affecting motility or sperm viability (data not shown). Inhibition of IDH (since 5 mM oxalomalate) significantly prevented AR ($p < .05$) (Figure 4b), without affecting motility or sperm viability (data not shown). With 10 mM of hydroxymalonate (specific inhibitor of MDH), the AR was completely inhibited ($p < .05$, Figure 4c) without affecting motility or sperm viability (data not shown).

4 | DISCUSSION

The current understanding of the processes that are involved in the acquisition of the sperm ability to fertilize oocytes includes two sequential processes: capacitation and acrosome reaction that encompasses several key components: the presence of energy substrates

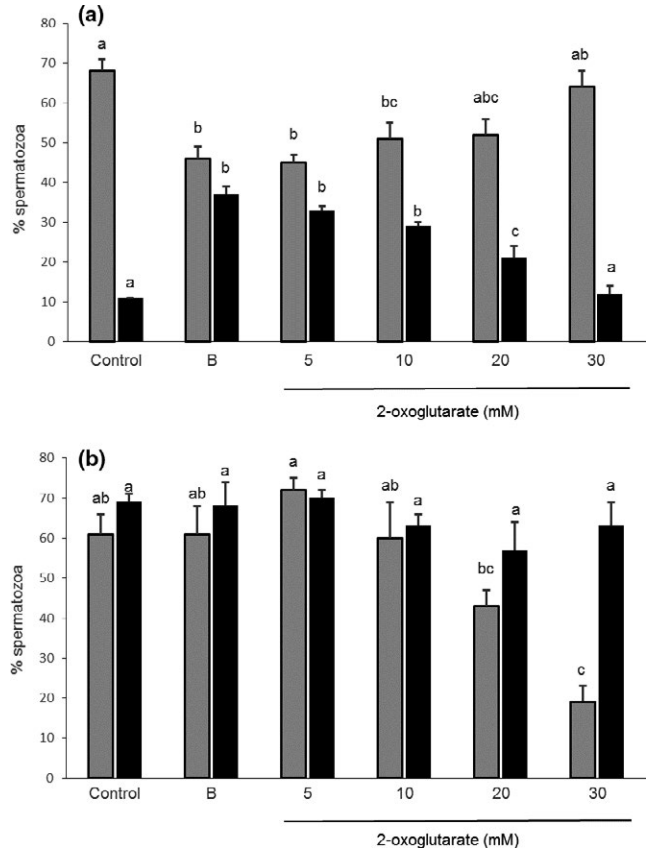

FIGURE 1 (a) Effect of 2-oxoglutarate on bicarbonate-induced capacitation of boar spermatozoa. Percentages of spermatozoa displaying F (▭) and B (■) CTC patterns. C: control; B: bicarbonate; 2-oxoglutarate: bicarbonate + different concentrations of 2-oxoglutarate. Means ± *SEM* of five replicates. For each parameter, the superscripts a-c indicate significant differences ($p < .05$). (b) Effect of 2-oxoglutarate on motility and viability of bicarbonate-induced capacitation of boar spermatozoa. Percentages of motile (▭) and viable (■) spermatozoa. C: control; B: bicarbonate; 2-oxoglutarate: bicarbonate + different concentrations of 2-oxoglutarate. Means ± *SEM* of five replicates. For each parameter, the superscripts a-c indicate significant differences ($p < .05$)

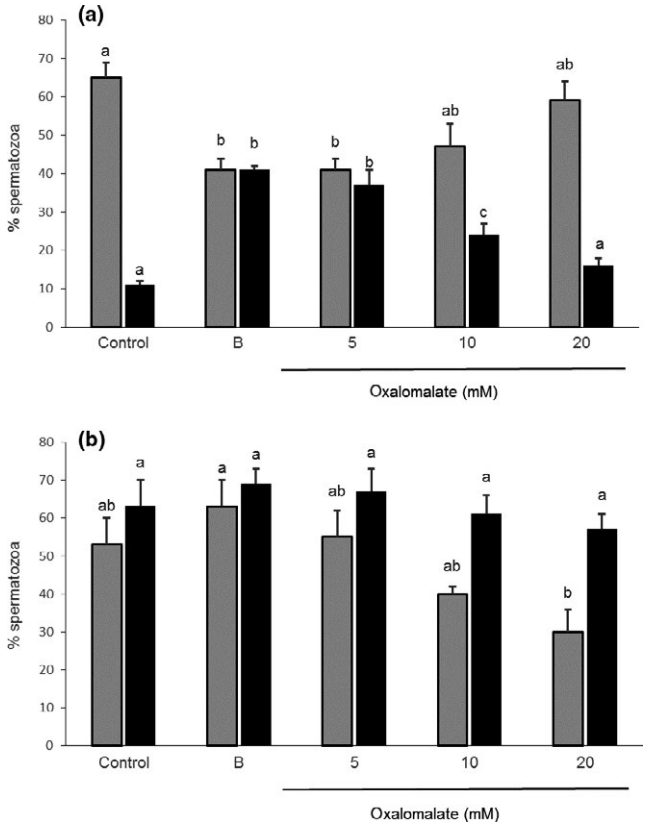

FIGURE 2 (a) Effect of oxalomalate on bicarbonate-induced capacitation of boar spermatozoa. Percentages of spermatozoa displaying F (▭) and B (■) CTC patterns C: control; B: bicarbonate; oxalomalate: bicarbonate + different concentrations of oxalomalate. Means ± *SEM* of five replicates. For each parameter, the superscripts a-c indicate significant differences ($p < 0.05$). (b) Effect of oxalomalate on motility and viability during bicarbonate-induced capacitation of boar spermatozoa. Percentages of motile (▭) and viable (■) spermatozoa. C: control; B: bicarbonate; oxalomalate: bicarbonate + different concentrations of oxalomalate. Means ± *SEM* of five replicates. For each parameter, the superscripts a, b indicate significant differences ($p < .05$)

necessary for ATP production; intracellular ion concentration and pH changes; cholesterol efflux and membrane fusion (Yanagimachi, 1994). Unfortunately, and despite vast amount of knowledge accumulated by many investigators in the past 20 years, there are several commonplaces regarding sperm energy metabolism, which, in fact, obstruct an optimal practical application of this knowledge (Rodríguez-Gil, 2013).

In this work, we have demonstrated the activity of PFK, IDH-NAD, IDH-NADP and MDH enzymes in boar spermatozoa. The addition of 30 mM oxoglutarate, specific inhibitor of the PFK, entirely blocked sperm capacitation and significantly diminished sperm motility, confirming the participation of this enzyme in bicarbonate-induced capacitation of porcine spermatozoa and the role of glycolysis in the generation of the energy necessary for this process. Interestingly, 10 mM of oxoglutarate prevented the follicular fluid-induced acrosome but failed to modify sperm motility. These results complements the findings of Kamp et al. (2007), who demonstrated the presence of this enzyme in boar spermatozoa and confirmed glycolytic activity in the flagellum and around the acrosome. Capacitation also involves activation of the sAC/cAMP/PKA pathway that in turn stimulates protein tyrosine phosphorylation (Vadnais, Galantino-Homer, & Althouse, 2007). The energy obtained by glycolysis can accomplish the need for ATP for cell signalling as proposed by Travis et al. (2001) for mouse spermatozoa.

The presence of IDH-NADP activity was demonstrated in bull spermatozoa and its inhibitor, oxalomalate, prevented sperm capacitation, suggesting an important role for this dehydrogenase to activate bull spermatozoa (O'Flaherty, Beorlegui, & Beconi, 2006). In agreement with this result in bovine spermatozoa, the addition of 20 mM oxalomalate inhibited porcine sperm capacitation, confirming the participation of this enzyme in bicarbonate-induced capacitation and the role of the Krebs cycle and/or the H+ equivalents in the generation of the energy or the signalling components necessary for this process. Oxalomalate inhibited not only NADP-dependent isoenzyme activity

(73 ± 2% of inhibition) but also NAD isoenzyme activity (81 ± 4 of inhibition), contrary to what observed by Adinolfi, Moratti, Olezza, and Ruffo (1969). The NADP-IDH reached on average 20-fold more enzymatic units than the isoenzyme NAD dependent. This result confirms the importance of the isocitrate dehydrogenation for energy production independently of the Krebs cycle flow, as it has been demonstrated in tissues that possess a constant energy supply, as heart and red skeletal muscle (Alp et al., 1976). This has also been demonstrated in gametes, bovine oocyte (Cetica, Pintos, Dalvit, & Beconi, 2003) and rat spermatozoa (Brooks, 1978).

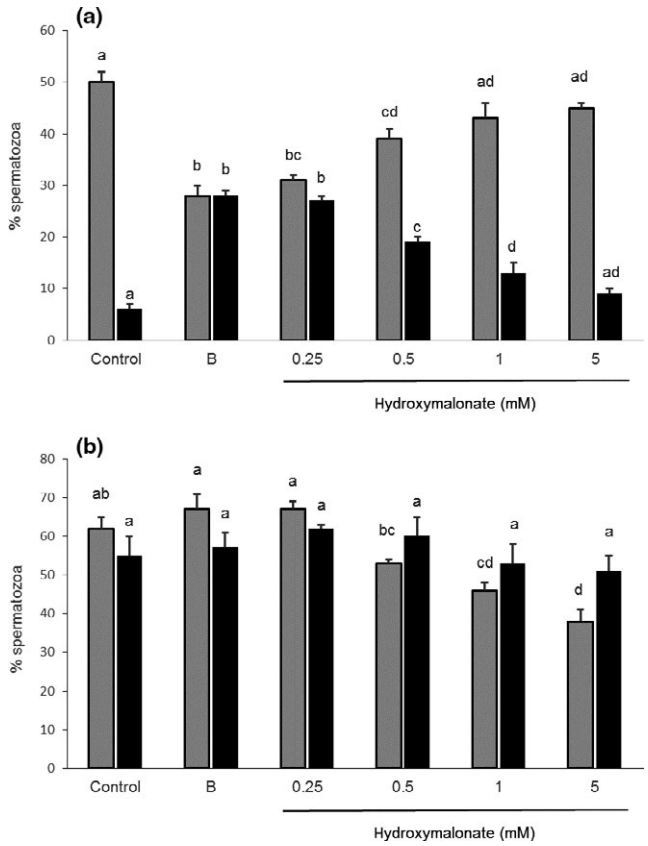

FIGURE 3 (a) Effect of hydroxymalonate on bicarbonate-induced capacitation of boar spermatozoa. Percentages of spermatozoa displaying F (▭) and B (■) CTC patterns C: control; B: bicarbonate; hydroxymalonate: bicarbonate + different concentrations of hydroxymalonate. Means ± *SEM* of five replicates. For each parameter, the superscripts a-d indicate significant differences ($p < .05$). (b) Effect of hydroxymalonate on motility and viability during bicarbonate-induced capacitation of boar spermatozoa. Percentages of motile (▭) and viable (■) spermatozoa. C: control; B: bicarbonate; hydroxymalonate: bicarbonate + different concentrations of hydroxymalonate. Means ± *SEM* of five replicates. For each parameter, the superscripts a-d indicate significant differences ($p < .05$)

Oxidation of cytosolic NADH in the mitochondrial respiratory chain is important in the production of energy in many cell types (Burgos, Coronel, de Burgos, Rovai, & Blanco, 1982; Gerez de Burgos, Burgos, Montamat, Moreno, & Blanco, 1978). Cytosolic NADH cannot traverse the mitochondrial membranes, so the reducing equivalents of cytosolic NADH are transferred to the mitochondria by shuttle systems. Malate dehydrogenase (MDH) is a main component of this shuttle and performs a key function in the passage of reduction equivalents through the internal mitochondrial membrane as a result of the presence of cytosolic and mitochondrial isoenzymes (Clarenburg, 1992). Enzymatic activity was high (approximately two-fold of PFK and IDH-NADP activity), suggesting that this activity can be linked not only with the Krebs cycle for oxidative energy supply but also with the malate–aspartate shuttle system. In tissues such as heart, liver and kidney, with a high capacity to metabolize lactate, the shuttle system is malate–aspartate (Dawson, 1979; Lehninger, Nelson, & Cox, 1997). This would be consistent with the high activity of lactate dehydrogenase found in porcine spermatozoa (Medrano et al., 2006). Moreover, in our experimental model, hydroxymalonate produced a complete blockage of sperm capacitation and a dose-dependent decrease in sperm motility during bicarbonate-induced capacitation, indicating that this enzyme plays a pivotal role in the mechanism that leads to capacitation in porcine spermatozoa.

It is noteworthy that many investigators indicate as an absolute fact that the energy obtained through the mitochondrial respiration is, under all conditions, absolutely necessary for the maintenance of sperm motility in all species (Folgero, Bertheussen, Lindal, Torbergsen, & Oian, 1993; Ford & Harrison, 1985; Halangk, Bohneback, & Kunz, 1985; Nevo, Polge, & Frederick, 1970; Ruız-Pesini et al., 1998), despite the fact that the same investigators maintain the absolute preeminence of glycolysis to obtain sperm energy, without realizing the contradiction in terms of energy that the simultaneous assumption

TABLE 3 Effect of the presence of oxoglutarate (20 mM), oxalomalate (10 mM) or hydroxymalonate (5 mM) during bicarbonate-induced capacitation on the percentages of true acrosome reaction induced by follicular fluid (30%), motility and sperm viability

	Acrosome Reaction (%)	Motility (%)	Viability (%)
Control	2 ± 1[a]	57 ± 2[a]	47 ± 3[a]
Bicarbonate/Follicular fluid	16 ± 2[b]	58 ± 3[a]	48 ± 4[a]
Bicarbonate + Oxoglutarate (20 mM)/Follicular fluid	7 ± 3[c]	45 ± 4[a]	44 ± 4[a]
Bicarbonate + Oxalomalate (10 mM)/Follicular Fluid	7 ± 3[c]	46 ± 4[a]	48 ± 3[a]
Bicarbonate + Hydroxymalonate (5 mM)/Follicular fluid	8 ± 1[c]	47 ± 1[a]	46 ± 1[a]

Values are means ± *SEM* of five replicates. For each parameter, the superscripts a-c indicate significant differences ($p < .05$).

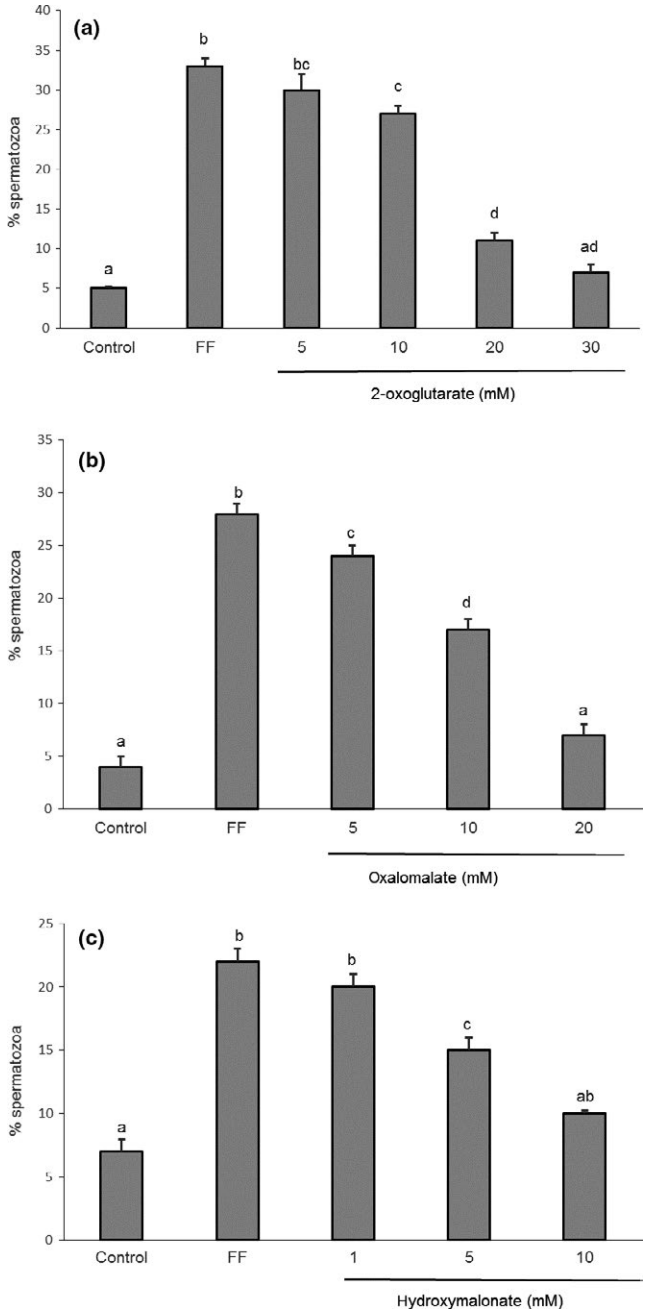

FIGURE 4 (a) Effect of oxoglutarate on follicular fluid-induced acrosome reaction of previously capacitated boar spermatozoa. C: control; FF: follicular fluid; 2-oxoglutarate: bicarbonate + different concentrations of 2-oxoglutarate. Means ± *SEM* of five replicates. The superscripts a-d indicate significant differences ($p < .05$). (b) Effect of oxalomalate on follicular fluid-induced acrosome reaction of previously capacitated boar spermatozoa. C: control; FF: follicular fluid; oxalomalate: bicarbonate + different concentrations of oxalomalate. Means ± *SEM* of five replicates. The superscripts a-d indicate significant differences ($p < .05$). (c) Effect of hydroxymalonate on follicular fluid-induced acrosome reaction of previously capacitated boar spermatozoa. C: control; FF: follicular fluid; hydroxymalonate: bicarbonate + different concentrations of hydroxymalonate. Means ± *SEM* of five replicates. The superscripts a-d indicate significant differences ($p < .05$)

of both principles implies (Rodríguez-Gil, 2013). In this work, we demonstrated that the addition of oxoglutarate, oxalomalate or hydroxymalonate (inhibitors of PFK, IDH and MDH) produces a dose-dependent decrease in sperm motility during capacitation but failed to modify sperm motility during the acrosome reaction. But these compounds prevented the follicular fluid-induced acrosome reaction, suggesting the participation of glycolysis, the Krebs cycle and the malate/aspartate shuttle in the acrosome reaction process. The role of glycolysis and the Krebs cycle–oxidative phosphorylation in the regulation of sperm motility remains a point of discussion. Whereas equine spermatozoa rely primarily on oxidative phosphorylation to generate the energy required for motility (Davila et al., 2016), other species utilize mostly glycolysis to provide ATP for flagellar propulsion (Ferramosca & Zara, 2014; Mukai & Okuno, 2004; Nascimento et al., 2008). Anyway, future studies are necessary to deep in the knowledge of the mechanisms involved in the generation of the energy not only for motility, but also capacitation and acrosome reaction processes in spermatozoa of the different mammal species.

In conclusion, we have determined the activity of phosphofructokinase, isocitrate dehydrogenase and malate dehydrogenase in porcine spermatozoa and our results demonstrate the participation of these enzymes in bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction, contributing to elucidate the metabolic pathways involved in the mechanisms that produce the energy necessary for these physiological processes in porcine spermatozoa.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Elizabeth Breininger performed the experiments to evaluate enzymatic activity, the analysis and interpretation of data, and drafted the manuscript. Denise Dubois and Vanina Eliana Pereyra performed the experiments about in vitro capacitation and acrosome reaction induction. Pablo Cristian Rodriguez and María Mercedes Satorre contributed to perform the experiments to evaluate sperm functionality and enzymatic activity, and participates in the analysis and interpretation of data. Pablo Cetica designed the experiments, contributed to the analysis and interpretation of data, and drafted the article.

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