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Role of amino acids and endogenous lipids in sperm capacitation of porcine spermatozoa

María Mercedes Satorre[1], Pablo Cristian Rodriguez[1], Elizabeth Breininger[1,2*], Pablo Daniel Cetica[1,2]

[1]Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal (INITRA, UBA), Buenos Aires, Argentina.
[2]Universidad de Buenos Aires - CONICET, Instituto de Investigaciones en Producción Animal (INPA, UBA-CONICET), Buenos Aires, Argentina.

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*Correspondence:

Corresponding author: Elizabeth Breininger
E-mail address: ebreininger@fvet.uba.ar

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ABSTRACT

The role of oxidative substrates and metabolic pathways in providing energy for porcine sperm capacitation is not yet fully understood. Our aim was to study the role of amino acids and endogenous lipids as oxidative substrates in porcine sperm capacitation, comparing them to classical oxidative substrates such as glucose and pyruvate. Sperm samples were incubated in capacitation media with or without classical oxidative substrates. Amino acids were added in the presence or absence of salicylate, an oxidative deamination inhibitor, to assess their potential as oxidative substrates. To evaluate endogenous lipid consumption, L-carnitine (a fatty acid β-oxidation inducer) and etomoxir (a fatty acid β-oxidation inhibitor) were utilized. Sperm motility, viability, capacitation, and ammonia production were evaluated for each treatment. In capacitation media without oxidative substrates, spermatozoa preserved motility and viability but failed to undergo capacitation. The addition of amino acids to the medium without oxidative substrates increased ammonia production but did not support sperm capacitation and diminished sperm motility. These effects were not observed when glucose and pyruvate were present in culture medium, although ammonia production was still increased. The addition of L-carnitine to the medium without oxidative substrates significantly improved sperm capacitation, whereas etomoxir had no effect. Boar spermatozoa have deamination activity, but amino acids by themselves cannot sustain sperm capacitation. Contrarily, catabolism of endogenous lipids can partially support sperm capacitation when they are the only oxidative substrates available. This study provides new insights into the role of metabolic pathways during porcine sperm capacitation and has significant implications for the development of assisted reproductive technologies in this species.

Introduction

Several studies have demonstrated that events associated with the fertilizing capacity of spermatozoa, like motility, capacitation, and acrosome reaction, directly depend on ATP production (Ho *et al*., 2002; Miki, 2007; Garrett *et al*., 2008). Sperm energy production has been mainly attributed to two different metabolic pathways, glycolysis and oxidative phosphorylation. The relative contribution of both metabolic pathways seems to be dependent on the animal species. It has been proposed that murine and porcine spermatozoa predominantly utilize glycolysis as energy source, while equine spermatozoa are more dependent on mitochondrial oxidative phosphorylation and bovine spermatozoa seem to have an intermediate behaviour, involving a combination of both metabolic pathways (Storey, 2008; Bucci *et al*., 2011; Rodríguez-Gil and Bonet, 2016). However, contradictory data have been published regarding ATP production in sperm cells, also in the same species, without reaching an agreement on the metabolic pathway that represents the primary source of energy for spermatozoa (Storey, 2008; Ramalho-Santos *et al*., 2009; Tourmente *et al*., 2015). To elucidate this controversy, some authors have proposed that spermatozoa might have versatility in adapting their metabolism depending on the available substrates, fertilization stage, or sperm environment (Ferramosca and Zara, 2014; Amaral, 2022).

Glucose is the main substrate in sperm capacitation media in the porcine species (Abeydeera and Day, 1997; Tardif *et al*., 2001). Several authors concluded that the most important metabolic pathway by which mature boar sperm obtain energy is glycolysis, while oxidative phosphorylation has a secondary role (Marin *et al*., 2003, Rodríguez-Gil and Estrada, 2013, Rodríguez-Gil and Bonet, 2016). In accordance, it was not observed a significant increase in oxygen consumption during porcine sperm capacitation (Ferramosca and Zara, 2014). On the other hand, it was detected enzymatic activity of key enzymes associated with tricarboxylic acid cycle and mitochondrial oxidative phosphorylation in porcine spermatozoa (Breininger *et al*., 2017; 2024), and it was observed oxygen uptake in cryopreserved boar sperm (Satorre *et al*., 2018).

Little is known about the use of amino acids and endogenous lipids in processes related to sperm fertilizing capacity, including sperm capacitation. Interestingly, both substrates must be oxidized in mitochondrial pathways in order to obtain energy (Nelson and Cox, 2021). Ciereszko *et al*. (1992) have detected activity of aminotransferases in porcine spermatozoa, while Zhu *et al*. (2020) described the presence of fatty acids β-oxidation enzymes in spermatozoa of this species. Oxidative deamination of amino acids can be inhibited by salicylate (Gould *et al*., 1963) and L-Carnitine acyl transferase 1 (CAT 1, which transfers the activated fatty acids into the mitochondrial matrix where the oxidation takes place) can be stimulated by L-carnitine and inhibited by etomoxir (Lombó *et al*., 2021; Schlaepfer and Joshi, 2020).

Studies about the use of amino acids or endogenous lipids as the only oxidative substrates during porcine sperm capacitation contribute to the understanding of their role in this process. Therefore, the aim of this work was to investigate the participation of amino acids and endogenous lipids as the unique oxidative substrates during boar sperm capacitation.

Materials and methods

Chemical reagents

Unless otherwise indicated, reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). MEM amino acid solution and MEM non-essential amino acid solution were from GIBCO® (Grand Island, NY, USA). Dextrose, sodium citrate, sodium bicarbonate, sodium

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chloride, sodium salicylate, EDTA, potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were from Merck® (Darmstadt, Germany).

Experimental Design

Experiment 1: To evaluate the participation of amino acids as the only oxidative substrates in the in vitro boar sperm capacitation, sperm samples were randomly divided into the following groups: T1) without oxidative substrates, T2) without oxidative substrates + HCO_3^- (capacitation inducer), T3) with the addition of amino acids + HCO_3^-, T4) with the addition of amino acids + HCO_3^- + 3 different concentrations of salicylate (to inhibit oxidative deamination), T5) with glucose+pyruvate (classical oxidative substrates), T6) with glucose+pyruvate + HCO_3^-, or T7) glucose+pyruvate + HCO_3^- + amino acids. Ammonia production was determined in the remaining media for each group. In each treatment, sperm motility, viability and capacitation were evaluated (n=5 for each treatment).

Experiment 2: To evaluate the participation of endogenous lipids as the unique oxidative substrates in the in vitro boar sperm capacitation, sperm samples were randomly divided into the following groups: T1) without oxidative substrates, T2) without oxidative substrates + HCO_3^-, T3) without oxidative substrates + HCO_3^-+ L-carnitine (to induce fatty acid ß-oxidation), T4) without oxidative substrates + HCO_3^-+ etomoxir (to inhibit fatty acid ß-oxidation), T5) with glucose+pyruvate, T6) or glucose+pyruvate + HCO_3^-. In each treatment, sperm motility, viability and capacitation were evaluated (n=5 for each treatment).

Sperm Samples

All procedures were approved following the guidelines of the Institutional Committee for Care and Use of Experimental Animals of the School of Veterinary Sciences, University of Buenos Aires (Protocol number: 2021/26). Boar samples used in this study were from a pool of five crossbred boars with proven fertility. These boars belonged to a controlled program of a local artificial insemination centre, and they were kept under uniform feeding and handling conditions during the entire study. Fresh samples with a minimum of 70% motile and 80% viable spermatozoa were refrigerated and stored at 16-18°C until use.

Sperm capacitation studies were carried out in TBM (Tris Buffer Medium), which contains glucose and pyruvate as oxidative substrates, Abeydeera and Day, 1997) with modifications according to the different treatments evaluated (see below).

Sperm Samples capacitation

Sperm capacitation with amino acids as oxidative substrates

The refrigerated boar sperm samples were tempered at 38°C for 10 min, centrifuged at 600 xg for 5 min and pellets were resuspended to a final concentration of 1.5-2.0 × 107 spermatozoa/mL and fractionated under different treatments: T1) without oxidative substrates (TBMns), T2) without oxidative substrates + HCO_3^- (TBMns+Bic: TBMns + 40mM sodium bicarbonate as capacitation inducer (Breininger *et al*., 2005), T3) with the addition of amino acids + HCO_3^- (TBMns+Bic+AA: TBMns+Bic+ 3% v/v MEM amino acids + 1% v/v MEM non-essential amino acids + 2 mM L-glutamine), T4) with the addition of amino acids + HCO_3^- + 3 different concentrations of salicylate (TBMns+Bic+AA+S1, S5 or S10: TBMns+Bic+AA + 1, 5 or 10 µM sodium salicylate as oxidative deamination inhibitor, respectively), T5) with glucose+pyruvate (TBM: classical oxidative substrates of TBM), T6) with glucose+pyruvate + HCO_3^- (TBM+Bic), or T7) with glucose+pyruvate + HCO_3^- + amino acids (TBM+Bic+AA). The samples were incubated at 38°C, 5% CO_2 in humidified air for 120 minutes (Satorre *et al*., 2009). Sperm motility, viability, capacitation, and ammonia production were evaluated for each treatment.

Sperm capacitation with endogenous lipids as oxidative substrates

Boar sperm samples were treated as described above and fractionated under different treatments: T1) without oxidative substrates (TBMns), T2) without oxidative substrates + HCO_3^- (TBMns+Bic), T3) without oxidative substrates + HCO_3^- + L-carnitine (TBMns+Bic+Carn: TBMns+Bic + 0,6 mg/mL L-carnitine as fatty acid ß-oxidation inducer, Aliabadi *et al*, 2013, T4) without oxidative substrates + HCO_3^- + etomoxir (TBMns+Bic+Etomoxir: TBMns+Bic + 0,05 mM etomoxir as fatty acid ß-oxidation inhibitor (Amaral *et al*. 2013), T5) glucose+pyruvate (TBM), or T6) glucose+pyruvate + HCO_3^- (TBM+Bic). The samples were incubated at 38°C, 5% CO_2 in humidified air for 120 minutes. Sperm motility, viability, and capacitation were evaluated for each treatment.

Sperm samples evaluation

Determination of sperm concentration

Sperm concentration was determined using a Neubauer chamber (Breininger *et al*, 2017).

Evaluation of sperm motility

Sperm motility was evaluated by light microscopy under 400x magnifications with a thermal stage at 37°C three times by the same observer (Satorre *et al*, 2012).

Evaluation of sperm viability

An aliquot of the sperm suspension was incubated with an equal volume of 0.25% Trypan blue in TALP at 37°C for 15 minutes. The mixture was then centrifuged at 600 ×g for 10 minutes to remove excess stain and subsequently fixed with 5% formaldehyde in PBS. Live spermatozoa appear unstained while dead ones appear blue (Breininger *et al*, 2017). Two hundred spermatozoa were evaluated for each treatment.

Evaluation of sperm in vitro capacitation

Sperm capacitation was evaluated through the modifications in fluorescence of chlortetracycline (CTC) patterns as described by Wang *et al*. (1995) and the ability of capacitated sperm to undergo true acrosome reaction (Breininger *et al*., 2005).

The CTC solution was freshly prepared daily by dissolving 500 µM CTC in a buffer containing 130 mM NaCl, 5 mM cysteine, and 20 mM Tris, pH 7.8. This solution was protected from light using foil. For the assay, 500 µL of the sperm suspension was mixed with an equal volume of the 500 µM CTC solution, and glutaraldehyde was added to achieve a final concentration of 0.1%. The prepared samples were then placed on a clean slide (Satorre and Breininger, 2021), and sperm capacitation was examined using an epifluorescence microscope (Jenamed 2, Carl Zeiss, Jena, Germany) at 400× magnification. Three fluorescence patterns were identified: F (fluorescent), indicating intact, non-capacitated sperm with uniform fluorescence across their surface; C (capacitated), signifying intact capacitated sperm with diminished fluorescence in the post-acrosomal region, reflecting capacitation; and AR (acrosome reacted), where sperm with a reacted acrosome exhibited loss of fluorescence in both the post-acrosomal and acrosomal regions, with fluorescence limited to the midpiece. The ability of capacitated spermatozoa to undergo true acrosome reaction was determined by incubating capacitated samples with 30% porcine follicular fluid (a physiological inducer of acrosome reaction, Breininger *et al*, 2017). True acrosome reaction was evaluated by trypan blue stain and differential-interferential contrast (DIC) patterns. Acrosome-reacted live spermatozoa appear unstained and with blurred acrosomes. Two hundred sperm were evaluated for each sample.

Evaluation of sperm ammonia production

Aliquots (100 µL) of the sperm suspensions were centrifuged and, in the supernatant, ammonia concentrations were determined by a spectrophotometric (340 ηm) assay based on NADPH oxidation by glutamate dehydrogenase (Ammonia enzymatic UV kit, 1009693, Wiener Lab, Alvarez et. al., 2012).

Statistical analysis

Percentages of sperm motility, viability, and capacitation, as well as ammonia production, were expressed as means ± SEM. 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. 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).

Results

Evaluation of the use of amino acids as only oxidative substrates for porcine sperm capacitation

To evaluate the role of amino acids as exclusive oxidative substrates during boar sperm capacitation, sperm cells were incubated under capacitation conditions without oxidative substrates (negative control), supplemented with amino acids, or amino acids combined with different concentrations of salicylate (an oxidative deamination inhibitor). Additionally, glucose and pyruvate (classical oxidative substrates) were used either alone or combined with amino acids as positive controls. Ammonia production in capacitation media was measured to evaluate sperm deamination activity. Capacitation was significantly higher in spermatozoa incubated in TBM (glucose+pyruvate) with the addition of bicarbonate as sperm capacitation inducer (TBM+Bic) respect to spermatozoa incubated in capacitation medium without oxidative substrates (TBMns+Bic; P<0.05; Figure 1). This finding demonstrates that oxidative substrates are needed as an energy source to sustain sperm capacitation. Although capacitation was not induced without classical oxidative substrates, spermatozoa preserved their motility and viability during incubation time (Table 1).

Table 1. Effect of inhibition or stimulation of amino acids oxidation on sperm motility and viability during bicarbonate-induced capacitation of porcine spermatozoa.

Treatment	Motility	Viability
TBMns	33.3±3.3[a]	45.8±5.9[ab]
TBMns+Bic	31.7±2.8[a]	43.5±3.7[abc]
TBMns+Bic+Aa	3.3±2.2[b]	36.2±5.5[abc]
TBMns+Bic+Aa+S1	4.2±2.8[b]	33.0±4.0[c]
TBMns+Bic+Aa+S5	5.8±1.4[b]	34.0±5.0[bc]
TBMns+Bic+Aa+S10	5.0±1.7[b]	33.7±5.0[bc]
TBM	33.3±4.4[a]	45.7±5.7[abc]
TBM+Bic	33.3±3.3[a]	46.8±6.8[a]
TBM+Bic+Aa	31.7±2.8[a]	46.3±5.7[ab]

TBMns: TBM medium without oxidative substrates; TBMns+Bic: TBMns with bicarbonate; TBMns+Bic+AA: TBMns with bicarbonate and amino acids; TBMns+Bic+AA+S1: TBMns with bicarbonate, amino acids and sodium salicylate 1 µM; TBMns+Bic+AA+S5: TBMns with bicarbonate, amino acids and sodium salicylate 5 µM; TBMns+Bic+AA+S10: TBMns with bicarbonate, amino acids and sodium salicylate 10 µM; TBM: TBM with glucose+pyruvate as oxidative substrates; TBM+Bic: TBM with bicarbonate; TBM+Bic+AA: TBM with bicarbonate and amino acids. Means ± SEM, n = 5. [a, b, c] Different letters indicate significant differences in the same column (P<0.05).

To examine whether amino acids could act as the only oxidative substrates during capacitation, amino acids were added to the capacitation medium without other oxidative substrates. Additionally, different

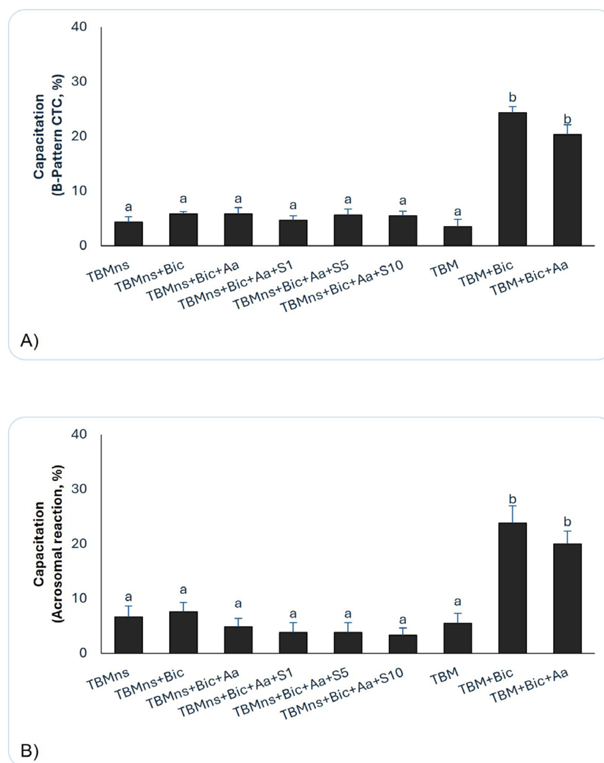

Figure 1. Effect of inhibition or stimulation of amino acids oxidation on bicarbonate-induced capacitation of porcine spermatozoa. Percentages of capacitated spermatozoa evaluated by CTC stain (a) and the capacity to respond to acrosome reaction induction evaluated by trypan blue/DIC (b). TBMns: TBM medium without oxidative substrates; TBMns+Bic: TBMns with bicarbonate; TBMns+Bic+AA: TBMns with bicarbonate and amino acids: TBMns+Bic+AA+S1: TBMns with bicarbonate, amino acids and sodium salicylate 1 µM; TBMns+Bic+AA+S5: TBMns with bicarbonate, amino acids and sodium salicylate 5 µM; TBMns+Bic+AA+S10: TBMns with bicarbonate, amino acids and sodium salicylate 10 µM; TBM: TBM with glucose+pyruvate as oxidative substrates; TBM+Bic: TBM with bicarbonate; TBM+Bic+AA: TBM with bicarbonate and amino acids. Means ± SEM, n = 5. [a, b] Different letters on bars indicate significant differences (P<0.05).

concentrations of sodium salicylate were also included in the medium containing only amino acids as substrates. Sperm ammonia production significantly increased in the presence of amino acids without other oxidative substrates (TBMns+Bic+AA; P<0.05), and the addition of salicylate diminished sperm ammonia production in a concentration-dependent manner (TBMns+Bic+AA+S1, S5 or S10; P<0.05), suggesting deamination activity by porcine sperm (Figure 2).

Spermatozoa incubated under capacitation conditions with amino acids as unique oxidative substrates (TBMns+Bic+AA) showed deamination activity but sperm capacitation was not observed under these conditions (Figure 1). In contrast, sperm capacitation was observed in media with amino acids in the presence of classical oxidative substrates (TBM+Bic+AA; P<0.05, Figure 1). Interestingly, sperm motility significantly diminished when amino acids were the only oxidative substrates present in the capacitation medium (TBMns+Bic+AA; P<0.05), but this effect was not observed when amino acids were included in the medium with classical oxidative substrates (TBM+Bic+AA; Table 1).

Evaluation of the use of endogenous lipids as unique oxidative substrates for porcine sperm capacitation

To evaluate the use of endogenous lipids as the unique oxidative substrates during capacitation of porcine spermatozoa, sperm cells were incubated under capacitation conditions without oxidative substrates (negative control) with the addition of L-carnitine, a fatty acid β-oxidation inducer, or etomoxir, a fatty acid β-oxidation inhibitor. Additionally, glucose and pyruvate (classical oxidative substrates) were added to the capacitation media as positive controls. In the presence of L-carnitine (TBMns+Bic+Carn), porcine spermatozoa increased the percentage of sperm capacitation respect to the medium without oxidative substrates (TBMns+Bic) (P<0.05, Figure 3), without reaching the values obtained in

the presence of classical oxidative substrates (TBM+Bic; P<0.05, Figure 3). These results suggest that endogenous lipids can partially sustain sperm capacitation in the porcine species. The addition of etomoxir to the capacitation medium (TBMns+Bic+Etom) did not affect sperm capacitation, motility or viability (TBMns+Bic; P<0.05, Figure 3 and Table 2).

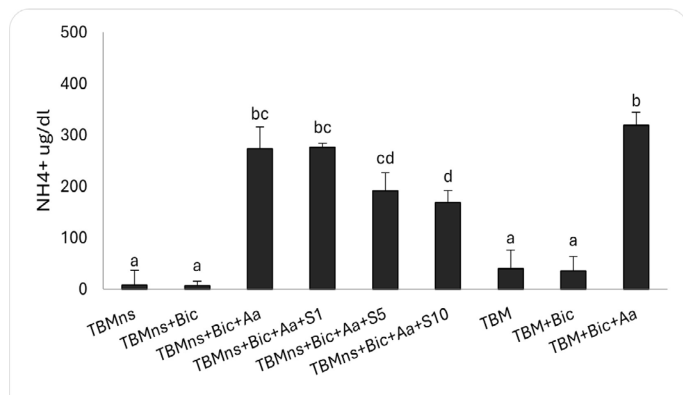

Figure 2. Effect of inhibition or stimulation of amino acids oxidation on ammonia (NH4+) production during bicarbonate-induced capacitation of porcine spermatozoa. TBMns: TBM medium without oxidative substrates; TBMns+Bic: TBMns with bicarbonate; TBMns+Bic+AA: TBMns with bicarbonate and amino acids; TBMns+Bic+AA+S1: TBMns with bicarbonate, amino acids and sodium salicylate 1 µM; TBMns+Bic+AA+S5: TBMns with bicarbonate, amino acids and sodium salicylate 5 µM; TBMns+Bic+AA+S10: TBMns with bicarbonate, amino acids and sodium salicylate 10 µM; TBM: TBM with glucose+pyruvate as oxidative substrates; TBM+Bic: TBM with bicarbonate; TBM+Bic+AA: TBM with bicarbonate and amino acids. Means ± SEM, n = 5. [a, b, c, d] Different letters on bars indicate significant differences (P<0.05).

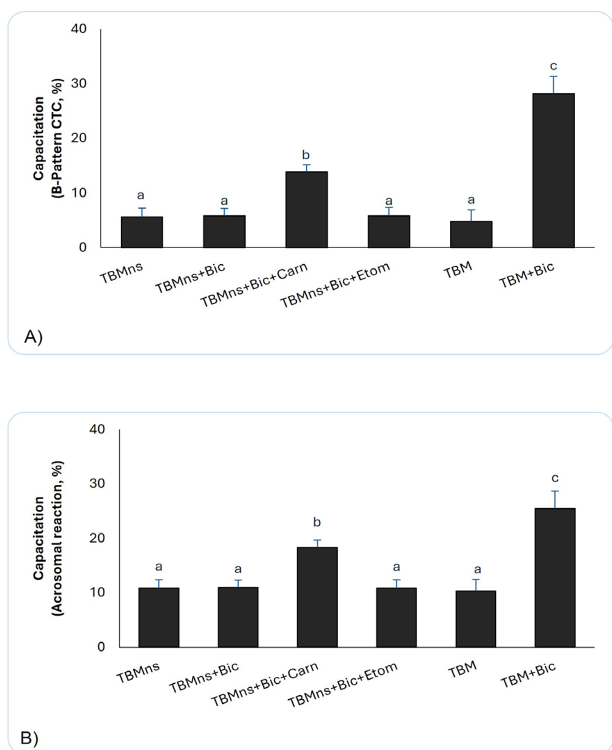

Figure 3. Effect of inhibition or stimulation of fatty acid β-oxidation on bicarbonate-induced capacitation of porcine spermatozoa. Percentages of capacitated spermatozoa evaluated by CTC stain (a) and the capacity to respond to acrosome reaction induction evaluated by trypan blue/DIC (b). TBMns: TBM medium without oxidative substrates; TBMns+Bic: TBMns with bicarbonate; TBMns+Bic+Carn: TBMns with bicarbonate and L-carnitine; TBMns+Bic+Etom: TBMns with bicarbonate and etomoxir; TBM: TBM with glucose+pyruvate as oxidative substrates; TBM+Bic: TBM with bicarbonate. Means ± SEM, n = 5. [a, b, c] Different letters on bars indicate significant differences (P<0.05).

Discussion

It has been reported that glucose is the main energy source for porcine spermatozoa, through its oxidation in glycolysis, producing the ATP required for its biological functions. Glycolysis has a pivotal role in the production of most of the energy produced in porcine spermatozoa, converting glucose to lactate, while just 5% of the consumed energy would have a mitochondrial origin (Marin *et al*., 2003, Rodríguez-Gil and Bonet, 2016). Although in bull and stallion spermatozoa an increase in oxygen uptake was registered during capacitation, in porcine spermatozoa this effect was not observed (Ferramosca and Zara, 2014). However, porcine spermatozoa did show an increase in oxygen consumption when exposed to succinate as a mitochondrial oxidative substrate (Satorre *et al*., 2018). Additionally, inhibiting enzymes related to the tricarboxylic acid cycle and mitochondrial respiratory chain, such as malate dehydrogenase, isocitrate dehydrogenase, and succinate dehydrogenase, led to a decrease in sperm capacitation and acrosome reaction in porcine spermatozoa (Breininger *et al*., 2017; 2024). Therefore, there is still a lack of information regarding the type of energy substrates involved in sperm function in the porcine species. The evaluation of oxidative substrates, like amino acids and lipids, which are metabolized in mitochondrial pathways (fatty acid β-oxidation, tricarboxylic acid cycle and respiratory chain), would contribute to the understanding of their role in functional events, like sperm capacitation.

Table 2. Effect of inhibition or stimulation of fatty acid β-oxidation on sperm motility and viability during bicarbonate-induced capacitation of porcine spermatozoa.

Treatment	Motility	Viability
TBMns	45.8±4.2^{a}	43.0±4.3^{a}
TBMns+Bic	40.0±3.3^{a}	42.0±2.7^{a}
TBMns+Bic+Carn	40.8±4.4^{a}	41.8±3.2^{a}
TBMns+Bic+Etom	40.8±4.4^{a}	41.0±3.7^{a}
TBM	45.8±4.2^{a}	45.0±3.7^{a}
TBM+Bic	40.8±4.4^{a}	43.2±4.5^{a}

TBMns: TBM medium without oxidative substrates; TBMns+Bic: TBMns with bicarbonate; TBMns+Bic+Carn: TBMns with bicarbonate and L-carnitine; TBMns+Bic+Etom: TBMns with bicarbonate and etomoxir; TBM: TBM with glucose+pyruvate as oxidative substrates; TBM+Bic: TBM with bicarbonate. Means ± SEM, n = 5. [a] Same letters indicate no significant differences in the same column (p > 0.05).

In our study, we observed that porcine spermatozoa incubated in a capacitation medium with bicarbonate, glucose and pyruvate (classical oxidative substrates) underwent capacitation and subsequent acrosome reaction, as it was also reported by other authors (Holt and Harrison, 2002; Litvin *et al*, 2003; Harrison and Gadella, 2005). However, when incubated in a medium without these classical oxidative substrates, they were unable to undergo capacitation and acrosome reaction, indicating that the presence of oxidative substrates is necessary to supply the energy required to sustain these processes. It is interesting to note that, although capacitation did not occur in the absence of exogenous oxidative substrates, sperm motility and viability were maintained, suggesting the use of endogenous substrates for this purpose. These results are in contrast with observations in mouse sperm which loses its motility in the absence of oxidative substrates in culture media (Romarowski *et al*., 2023) but in agreement with those observed in human sperm which maintains motility under these conditions (Amaral, 2022).

In order to evaluate the role of amino acids in porcine sperm capacitation, exogenous amino acids were included as the sole oxidative substrates in the capacitation medium, in the absence or presence of an oxidative deamination inhibitor (salicylate). Sperm deamination capacity was evaluated measuring ammonia production by spermatozoa. Our results indicate that porcine spermatozoa have deamination capacity as ammonia production was increased in those media where amino acids were present. This effect was confirmed by observing that the sperm ammonia production decreased in a dose-dependent manner in the presence of the oxidative deamination inhibitor. Despite the deamination capacity detected in porcine spermatozoa, the amino acids were not able to supply the energy needed to sustain sperm capacitation. Moreover, when amino acids were the only oxidative substrates, sperm motility significantly decreased, indicating a detrimental effect. Other authors have observed in the bovine species that the oxidation of amino acid by spermatozoa produces ammonia accumulation which would be deleterious

for sperm metabolism (Gregoire *et al*., 1961) and viability (Macmillan *et al*., 1972). Additionally, an excess of amino acids could be increasing the activity of sperm L-amino acid oxidases, generating an increase in reactive oxygen species production that could be deleterious for sperm function (Aitken *et al*., 2022). On the other hand, we observed that, when amino acids were added to the capacitation medium in the presence of glucose and pyruvate, sperm capacitation occurred and motility was maintained, despite the increase of ammonia production detected. These results suggest that the sperm function impairment is not dependent only on ammonia concentration and that it would be more related to the way in which amino acids are being used. More studies should be done in order to clarify this effect.

To evaluate the use of endogenous lipids as unique oxidative substrates in porcine sperm capacitation, we stimulated fatty acid β-oxidation by the addition of L-carnitine (CAT 1 activity inducer) to the capacitation medium without exogenous oxidative substrates. L-carnitine significantly increased capacitation in porcine spermatozoa, indicating that the energy necessary to support this process can be, at least partially, obtained from the catabolism of endogenous lipids. Anyway, the sperm capacitation rate obtained with the addition of L-carnitine did not reach the same values of those with classical oxidative substrates, suggesting that exogenous substrates are necessary to sustain the complete capacitation process. Due to the limited lipid stores in the spermatozoon, structural lipids would likely be used as energy source, as previously reported (Amaral, 2022; Weide *et al*, 2024). In accordance with our results, it has been recently observed that exogenous long chain fatty acids, oleic and palmitic acids, enhances ATP production in porcine spermatozoa by enhancing fatty acid β-oxidation (Zhu *et al*., 2020).

Although we have observed that capacitation did not occur in the medium without oxidative substrates in porcine spermatozoa, they maintained their motility and viability in this medium, suggesting the use of endogenous substrates for energy source. We evaluated the catabolism of endogenous lipids by using etomoxir, an inhibitor of fatty acid β-oxidation. Although higher concentrations of etomoxir showed a decrease in sperm motility in human sperm (Amaral *et al*, 2013), suggesting that the inhibition of fatty acid β-oxidation compromises its energy production, the addition of etomoxir did not affect porcine sperm motility and viability at the concentration used in this study.

Conclusion

As far as we know, this is the first study to evaluate the exclusive use of amino acids or endogenous lipids as oxidative substrates during boar sperm capacitation. Our findings provide new information about the role of metabolic pathways present in the mitochondria of porcine spermatozoa, contributing to the understanding of energy supply for sperm capacitation. Although porcine spermatozoa have the ability to deaminate exogenous amino acids, these compounds cannot be used as the only oxidative substrates to energetically sustain sperm capacitation, but endogenous lipids can partially support the energy required for this process in the absence of other exogenous oxidative substrates.

Acknowledgments

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Conflict of interest

The authors have no conflict of interest to declare.

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