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Participation of lactate dehydrogenase in capacitation and acrosome reaction of fresh and cryopreserved, with or without α-tocopherol, boar sperm*

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The aim of this work was to study the participation of lactate dehydrogenase (LDH) enzyme in capacitation and acrosome reaction in porcine sperm by determining its enzymatic activity and the effect of its inhibition on these processes. Semen samples, from four crossbred boars (Pietrain x Yorkshire) of proven fertility, 1-1.5 year of age, were analysed. The enzymatic activity of LDH was determined spectrophotometrically in extracts from fresh and cryopreserved, with and without α-tocopherol, porcine sperm. Besides, sperm suspensions were incubated in the presence of bicarbonate (40 mM), a well-known capacitation inducer, follicular fluid (30%), as an acrosome reaction inducer, and different concentrations of sodium oxamate, a specific inhibitor of the enzyme. The activity of LDH in enzymatic extracts of fresh, cryopreserved without or cryopreserved with α-tocopherol porcine sperm were 3.85±0.27, 0.7±0.04 and 1.73±0.23 U/10^{10} sperm, respectively ($p<0.05$). The addition of sodium oxamate inhibited capacitation or acrosome reaction in fresh and cryopreserved with or without α-tocopherol porcine sperm, affecting (at different concentrations) motility and sperm viability ($P<0.05$). Obtained results demonstrate the participation of LDH in bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction in fresh and cryopreserved porcine sperm.

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Capacitation is a preparation process of sperm to acquire the ability to fertilize mature oocytes that includes changes in plasma membrane fluidity, O_2 uptake, intracellular ionic concentration and the activity of several enzymes. This process has been first described as a biochemical and reversible process [Chang 1951] which enables the sperm to undergo the acrosome reaction (AR) [Yanagimachi 1994].

Efficient ATP generation by sperm is crucial for fertilization [Ho*et al.* 2002]. In a metabolomic study, it has been observed that approximately 95 % of the energy yielded by glucose in boar sperm is originated through the glycolytic pathway, while the remaining 5% is produced by the mitochondrial respiration [Marin *et al.* 2003].

The main product of the glycolytic pathway is pyruvate. Lactate dehydrogenase (LDH) converts pyruvate into lactate with the concomitant oxidation of NADH to NAD to be used again in the pathway, that is why this enzyme is essential for the continued production of energy by glycolysis [Li *et al.* 1989]. Energy metabolism of sperm is closely related to the activity of LDH in many species [Casano *et al.* 1991, Kohsaka *et al.* 1992, Jones 1997]. O'Flaherty *et al.* [2002] reported that LDH present in the cytosol and the plasma membrane of bovine sperm produces lactate, which is involved in capacitation of cryopreserved bovine sperm, while Duan and Goldberg [2003] confirmed LDH participation in mouse sperm capacitation. It has also been suggested that lactate produced by the sperm-specific mitochondrial LDH isoform could enter into the cytosol and generate NADH by means of the cytosolic isoenzyme, establishing the redox status required for sperm capacitation [O'Flaherty *et al.* 2005]. In boar sperm, it has been shown that the sperm-specific LDH isoform regulates lactate metabolism in these cells [Jones 1997].

Freezing-thawing processes alter membrane selective permeability [Phelps *et al.* 1999], decrease energy production [Evenson *et al.* 1982] and reduce sperm motility [De Leeuw *et al.* 1991]. Additionally, boar sperm is particularly susceptible to the peroxidative damage produced by cryopreservation [Cerolini *et al.* 2000]. Our group has demonstrated that the addition of α-tocopherol in the freezing extender exerts a protective effect on these events in cryopreserved boar sperm [Breininger *et al.* 2005 b, Satorre *et al.* 2007].

Taking into account that porcine sperm obtains energy mainly through glycolytic pathway, that the LDH enzyme plays a pivotal role in the mechanisms that allows spermatozoa to obtain the energy required for capacitation and acrosome reaction for subsequent fertilization, and that antioxidants, like α-tocopherol, protect boar sperm against cryopreservation damage, the aim of this work was to study the participation of LDH in capacitation and acrosome reaction in fresh and cryopreserved with or without α-tocopherol porcine sperm by determining its enzymatic activity and the effect of its inhibition on these processes.

Material and methods

Reagents

Unless otherwise indicated, reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). Orvus ES Paste (Equex-Paste) was from Minitub (Tiefenbach b. Landshut, Germany). Eosin yellowish and nigrosine from Mallinckrodt (St. Louis, MO, USA). Dextrose, sodium citrate, sodium bicarbonate, sodium chloride, EDTA, potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany).

Semen collection

Semen samples, from four crossbred boars (Pietrain x Yorkshire) of proven fertility, 1-1.5 year of age, were provided by the Porcine Productive Unit of the School of Veterinary Sciences (University of Buenos Aires). This Productive Unit replicates a small porcine production system in uniform and controlled feeding and handling conditions. All animal experiments were performed in accordance with the guidelines of the Institutional Committee for Care and Use of Experimental Animals of the School of Veterinary Sciences, University of Buenos Aires. Only samples with a minimum of 70 % motile and 80 % viable sperm were included in the study.

Sperm freezing

Each ejaculate was split into three portions. One for fresh samples (F) and two for cryopreserved with (α-t samples) or without the addition of 200 ug/ml of α-tocopherol (C samples) to freezing extender according to Pursel and Johnson´s protocol as described by Perez Colman *et al.* [2019]. The samples were frozen into straws of 0.5 mL and stored in liquid nitrogen at -196 ºC.

Evaluation of sperm motility and viability

Total sperm motility was evaluated, three times by the same observer, by light optical microscopy (400x magnification) and a thermal stage (37°C). The percentage of live sperm was determined by the supravital eosin/nigrosin technique [Pintado 2000]. At least 200 sperm were counted in each sample.

Determination of lactate dehydrogenase activity

Enzymatic extracts were prepared according to Breininger *et al*. [2017]. The LDH enzymatic activity was measured in a Shimadzu spectrophotometer model UV-160 (Shimadzu Corporation, Tokyo, Japan) according to the protocol described by Trinchero *et al.* [1993] with minimum modifications. The activity was measured (340 nm, 37 °C) for 10 minutes in 120 mM phosphate buffer pH 7.4 (Na_2HPO_4 85%: NaH_2PO_4 15%) supplemented with (assay concentrations) 0.2 mM pyruvate and 0.115 mM NADH. Sodium oxamate (SO) was used as specific enzymatic inhibitor of LDH. Enzymatic activity was expressed as units (U) per 10^{10} sperm. The U of LDH

was defined as the quantity of enzyme that catalysed the conversion reduction of 1 μmol of NADH per minute. The inhibition percentage was calculated as 100 - activity rate, while the activity rate = (U-of-samples-with-inhibitor / U-of-samples-without-inhibitor) x 100.

Sperm capacitation

Fresh and cryopreserved samples were capacitated according to Satorre *et al*. [2009] in the absence (negative control) or presence (positive control) of 40 mM sodium bicarbonate, and different SO concentrations. Sperm capacitation state was evaluated through the modifications in fluorescence of CTC patterns as described by Wang *et al*. [1995].

Acrosome reaction

Capacitated fresh or cryopreserved sperm were additionally incubated in the absence (negative control) or presence (positive control) of 30% follicular fluid [Breininger *et al.* 2005a] with the addition of different SO concentrations. True acrosomal reaction was evaluated by Trypan blue and Differential-Interferential Contrast (DIC) [O'Flaherty *et al.* 1997]. At least 200 spermatozoa were counted in each sample.

Statistical analysis

Percentages of motility, viability, sperm capacitation, true acrosome reaction and LDH activity (expressed as U per 10^{10} sperm) are given as mean and standard deviation. The quantitative data collected were analysed for assumption of normality by Shapiro-Wilk test and homogeneity of variances using the Levene test. When necessary, percentages of motility, viability, sperm capacitation and true acrosome reaction were normalized by arcsine transformation. Values were analysed by one-way ANOVA and post-hoc Bonferroni test. Differences were considered as statistically significant at $p<0.05$.

Results and discussion

A significantly lower activity of LDH was observed in both cryopreserved samples in comparison with the activity observed in fresh samples. Within α-t samples LDH activity reached 2.5 times higher activity level in comparison with the control ($p<0.05$). The activity of LDH in the enzymatic extracts of fresh samples was almost completely inhibited (93%) by the addition of 50 mM of SO. On the other hand, in frozen sperm, the SO addition much lower then 5 mM was sufficient to achieve a high inhibition of the enzyme activity (88% and 85% for α-t or C samples, respectively, Tab. 1).

In this paper, LDH enzyme activity was considerably lower in cryopreserved porcine spermatozoa compared with fresh sperm, but this effect was partially attenuated by the α-tocopherol supplementation. The loss of enzymatic activity of

Table 1. Enzymatic activity of lactate dehydrogenase (LDH)

	LDH activity	10 mM sodium oxamate		25 mM sodium oxamate		50 mM sodium oxamate	
		activity	inhibition (%)	activity	inhibition (%)	activity	inhibition (%)
Fresh porcine spermatozoa	3.85±0.27^{a,1}	2.44±0.26^{b}	37±6	0.88±0.18^{b}	75±4	0.27±0.07^{c}	93±2
	LDH activity	0.5 mM sodium oxamate		1 mM sodium oxamate		5 mM sodium oxamate	
		activity	inhibition (%)	activity	inhibition (%)	activity	inhibition (%)
Cryopreserved porcine sperm	0.70±0.04^{a,2}	0.38±0.05^{b}	46±7	0.19±0.05^{bc}	73±7	0.11±0.02^{c}	85±3
	LDH activity	0.5 mM sodium oxamate		1 mM sodium oxamate		5 mM sodium oxamate	
		activity	inhibition (%)	activity	inhibition (%)	activity	inhibition (%)
Cryopreserved with α-tocopherol porcine sperm	1.73±0.23^{a,3}	1.00±0.14^{b}	40±7	0.46±0.11^{bc}	73±6	0.23±0.11^{c}	88±5

Effect of the addition of sodium oxamate on LDH activity in fresh, C or α-t boar sperm; Enzymatic activity is expressed in U per 10^{10} sperm (means ± se of five replicates).
$^{1, 2, 3}$ Indicate significant differences ($p<0.05$) for LDH activity without sodium oxamate.
$^{a, b, c}$ Indicate significant differences ($p < 0.05$), for each treatment.

several enzymes caused by sperm cryopreservation has already been reported [Jones and Mann 1977, Glogowski *et al.* 1996, Babiak *et al.* 2001, Chauhan *et al.* 2009]. Loss of SOD activity in bovine spermatozoa was recorded by Beconi *et al.* [1991], who also demonstrated a protective effect of α-tocopherol on the activity of this enzyme.

SO has already been used as a competitive inhibitor of LDH activity in mouse [Odet *et al.* 2011] and human [Hereng *et al.* 2011] spermatozoa. In the current study

we evaluated the effect of different concentrations of this inhibitor on LDH activity (in extracts of fresh, frozen or α-t porcine spermatozoa). The concentrations of SO required for significant inhibition of LDH activity in cryopreserved spermatozoa samples were lower than the concentrations required for fresh sperm samples, probably due to the decrease of LDH activity detected in the frozen samples which resulted from possible membrane alterations caused by the freezing/thawing procedure. Interestingly, we observed highest levels of LDH inhibition in porcine fresh sperm samples at lower concentrations than the concentrations previously applied by Odet *et al.* [2011] in mouse sperm, which resulted probably from different characteristics of the sperm membrane between species.

In fresh samples, none of the SO concentrations evaluated significantly lowered capacitation levels comparing to the positive control – meanwhile acrosome reaction was inhibited starting at 25 mM of SO (Fig. 1). Neither motility nor sperm viability were affected by the addition of the LDH inhibitor during *in vitro* capacitation, but 50 mM of SO affected motility and sperm viability during acrosome reaction (45% and 36% of inhibition in comparison with to the positive control, respectively).

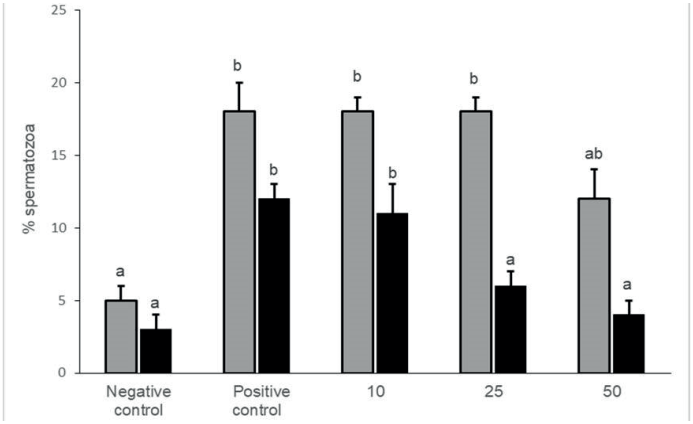

Fig. 1. Effect of sodium oxamate on bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction in fresh porcine sperm. Negative control (no inducers); Positive control: 40 mM bicarbonate or 30% follicular fluid; 10; 25 and 50: positive control + sodium oxamate (10 mM; 25 mM or 50 mM). Percentages of capacitated (grey bars) or acrosome reacted (black bars) spermatozoa (mean±se) of five replicates; a, b - for both parameters, different letters indicate significant differences between treatments ($p<0.05$).

The addition of 25 mM of SO prevented the follicular fluid-induced acrosome reaction without affecting capacitation, motility or sperm viability in fresh samples, suggesting differential participation of this enzyme in glycolytic activity needed for energy generation required for these processes. These results are in agreement with the findings of O'Flaherty *et al*. [2002] and Duan and Goldberg [2003] in cryopreserved bovine and fresh mouse sperm, respectively.

In frozen samples, capacitation and acrosome reaction were inhibited by the addition of 1 mM and 0.5 mM SO, respectively (Fig. 2). During capacitation, 1 mM

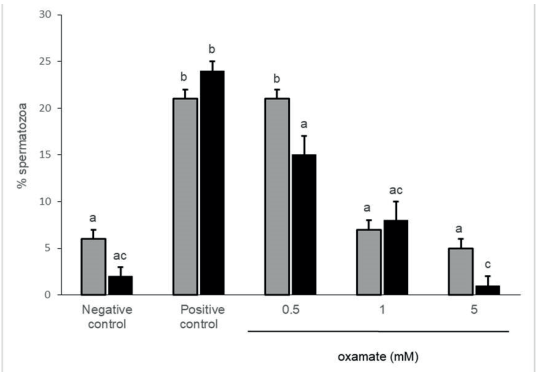

Fig. 2. Effect of sodium oxamate on bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction in cryopreserved porcine sperm. Percentages of capacitated (grey bars) or acrosome reacted (black bars) spermatozoa (mean+se) of five replicates. a, b, c - for both parameters, different letters indicate significant differences between treatments ($p<0.05$).

concentration diminished motility and sperm viability (75% and 24% of inhibition in comparison to the positive control, respectively). During acrosome reaction, motility and sperm viability were hightly diminished by a lower concentration of 0.5 mM (33% and 19% of inhibition in comparison to the positive control, respectively).

As observed in frozen samples, capacitation and acrosome reaction in α-t samples were inhibited by the addition of 1 mM and 0.5 mM SO, respectively (Fig. 3). During capacitation, 1 mM concentration decreased motility and sperm viability (44% and 31% of inhibition in comparison to the positive control). In follicular fluid-induced acrosome reaction, motility and sperm viability were decreased by 0.5 mM (29% and 10% of inhibition in comparison to the positive control, respectively).

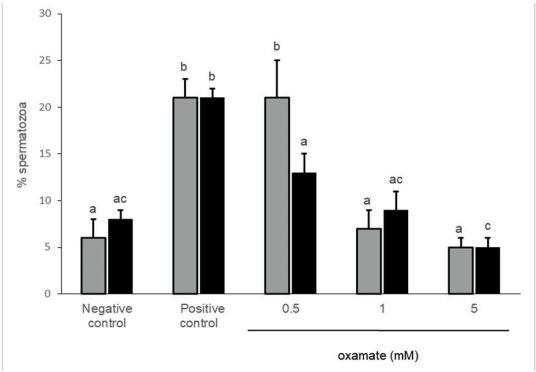

Fig. 3. Effect of sodium oxamate on bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction in cryopreserved with α-tocopherol porcine sperm. Negative control; Positive control: 40 mM bicarbonate or 30% follicular fluid; 0.5; 1 and 5: positive control + sodium oxamate (0.5 mM; 1 mM or 5 mM). Percentages of capacitated (grey bars) or acrosome reacted (black bars) spermatozoa (mean+SEM) of five replicates. a, b, c For both parameters, different letters indicate significant differences between treatments ($p<0.05$).

With respect to cryopreserved sperm, the addition of 1 mM and 0.5 mM SO significantly diminished bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction, respectively, affecting motility and sperm viability. These results confirm the participation of LDH in capacitation and acrosome reaction processes; but probably because of the sperm membrane damage due to freezing-thawing procedures, the concentration of SO needed to cause detectable inhibition the enzyme is considerably lower than that required for fresh sperm. The lowering of motility and sperm viability observed may be also related to this effect, but motility was less affected in cryopreserved sperm with α-tocopherol as we reported previously [Satorre *et al*. 2007].

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, despite the fact that the same investigators maintain the absolute pre-eminence of glycolysis to obtain sperm energy [Nevo *et al.* 1970, Folgerø *et al.* 1993, Ruiz-Pesini *et al.* 1998], without realizing the contradiction in terms of energy which the simultaneous assumption of both principles implies [Rodríguez-Gil 2013]. We demonstrated that the inhibition of LDH prevented capacitation and acrosome reaction, suggesting the pivotal role of the glycolytic pathway in the generation of the energy required for those processes. However, the role of glycolysis, Krebs cycle and oxidative phosphorylation in the regulation of sperm motility remains a point of discussion. Additionally, we observed that motility was impaired in the presence of SO depending on the concentration of the inhibitor used, if fresh or cryopreserved sperm were analysed, and if sperm capacitation or acrosome reaction was evaluated. Anyway, future studies are necessary to deepen the knowledge of the mechanisms involved in the generation of the energy responsible not only for motility, but also for capacitation and acrosome reaction.

In conclusion, our results demonstrate the participation of lactate dehydrogenase enzyme in bicarbonate-induced capacitation and follicular fluid-induced acrosome reaction in fresh and cryopreserved porcine sperm, deepening the knowledge about the metabolic pathways that produce the energy necessary for these physiological processes in this species.

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REFERENCES

1. BABIAK I., GLOGOWSKI J., GORYCZKO K., DOBOSZ S., KUZMINSKI H., STRZEZEK J., DEMIANOWICZ W., 2001 – Effect of Extender Composition and Equilibration Time on Fertilization Ability and Enzymatic Activity of Rainbow Trout Cryopreserved Spermatozoa. ***Theriogenology*** 56 (1), 177-192.
2. BECONI M.T., AFFRANCHINOM.A., SCHANGL.M., BEORLEGUIN.B., 1991 – Influence of Antioxidants on SOD Activity in Bovine Sperm. ***Biochemistry International*** 23(3), 545-553.
3. BREININGER E., BEORLEGUI N.B., BECONI M.T., 2005a – DifferentialEffectofFollicular Fluid and the Zona Pellucidaonthe Acrosomal Reaction in CryopreservedPorcine Semen. VI Simposio Internacional de Reproducción Animal (IRAC), Córdoba (Argentina), 24-26 junio 2005.

4. BREININGER E., BEORLEGUI N.B., O'FLAHERTY C.M., BECONI M.T., 2005b – Alpha-Tocopherol Improves Biochemical and Dynamic Parameters in Cryopreserved Boar Semen. ***Theriogenology*** 63(8), 2126-2135.
5. BREININGER E., DUBOIS D., PEREYRA V.E., RODRIGUEZ P.C., SATORRE M.M., CETICA P.D., 2017 – Participation of phosphofructokinase, malate dehydrogenase and isocitrate dehydrogenase in capacitation and acrosome reaction of boar spermatozoa. ***Reproduction in Domestical Animal*** 52(5), 731-740.
6. CASANO R., ORLANDO C., SERIO M., FORTI G., 1991 – LDH and LDH-X Activity in Sperm from Normospermic and Oligozoospermic Men. ***International Journal of Andrology*** 14(4), 257-263.
7. CEROLINI S., MALDJIAN A., SURAI P., NOBLE R., 2000 – Viability, Susceptibility to Peroxidation and Fatty Acid Composition of Boar Semen during Liquid Storage. ***Animal Reproduction Science*** 58, 99-111.
8. CHANG M.C., 1951 – Fertilizing Capacity of Spermatozoa Deposited into the Fallopian Tubes. ***Nature*** 168, 697-698.
9. CHAUHAN M.S., KAPILA R., GANDHI K.K., ANAND S.R., 2009 – Acrosome Damage and Enzyme Leakage of Goat Spermatozoa during Dilution,Cooling and Freezing. ***Andrologia*** 26,21-26.
10. DE LEEUW F.E., CALENBRANDER B., VERKLEIJ A.J., 1991 – The Role Membrane Damage Plays in Cold Shock and Freezing Injury. In Boar Semen Preservation II, ed.Rath D. Johnson LA. Berlin: Paul Parey Scientific Publishers. Pages 95-104.
11. DUAN C., GOLDBERG E., 2003 – Inhibition of Lactate Dehydrogenase C4 (LDH-C4) Blocks Capacitation of Mouse Sperm in Vitro. ***Cytogenetic and Genome Research*** 103, 352-359.
12. EVENSON D.P., DARZYNKIEWICZ Z., MELAMED M.R., 1982 – Simultaneous Measurement by Flow Cytometry of Sperm Cell Viability and Mitochondrial Membrane Potential Related to Cell Motility. ***The Journal of Histochemistry and Cytochemistry,*** 30(3), 279-280.
13. FOLGERØ T., BERTHEUSSEN K., LINDAL S., TORBERGSEN T., OIAN P., 1993 – Mitochondrial Disease and Reduced Sperm Motility. ***Human Reproduction,***8(11), 1863-1868.
14. GLOGOWSKI J., BABIAK I., GORYCZKO K., DOBOSZ S., 1996 – Activity of Aspartate Aminotransferase and Acid Phosphatase in Cryopreserved Trout Sperm. ***Reproduction, Fertility and Development,*** 8(8), 1179-1184.
15. HERENG T.H., ELGSTØEN K.B.P., CEDERKVIST F.H., EIDE L., JAHNSEN T., SKÅLHEGG B.S., ROSENDAL K.R., 2011 – Exogenous Pyruvate Accelerates Glycolysis and Promotes Capacitation in Human Spermatozoa. ***Human Reproduction***, 26(12), 3249-3263.
16. HO H.C., GRANISH K.A., SUAREZ S.S., 2002 – Hyperactivated Motility of Bull Sperm Is Triggered at the Axoneme by Ca2 and Not cAMP. ***Developmental Biology,*** 250,208-217.
17. JONES R., MANN T., 1977 – Damage to Ram Spermatozoa by Peroxidation of Endogenous Phospholipids. ***Reproduction,***50(2), 261-268.17.
18. Jones A.R. 1997 – Metabolism of Lactate by Mature Boar Spermatozoa. ***Reproduction, Fertility and Development,*** 9(2), 227-232.
19. KOHSAKA T., TAKAHARA H., TAGAMI S., SASADA H., MASAKI J., 1992 – A New Technique for the Precise Location of Lactate and Malate Dehydrogenases in Goat, Boar and Water Buffalo Spermatozoa Using Gel Incubation Film. ***Journal of Reproduction and Fertility***, 95(1), 201-209.
20. LI S.S.-L., O'BRIEN D.A., HOU E.W., VERSOLA J., ROCKETT D.L., EDDY E.M., 1989 – Differential Activity and Synthesis of Lactate Dehydrogenase Isozymes A (Muscle), B (Heart), and C (Testis) in Mouse Spermatogenic Cells. ***Biology of Reproduction***, 40(1), 173-180.
21. MARIN S., CHIANG K., BASSILIAN S., LEE W.-N.P., BOROS L.G., FERNÁNDEZ-NOVELL J.M., CENTELLES J.J., MEDRANO A., RODRIGUEZ-GIL J.E., CASCANTE M., 2003 – Metabolic Strategy of Boar Spermatozoa Revealed by a Metabolomic Characterization. ***FEBS Letters***, 554(3),342-346.

22. NEVO A.C., POLGE C., FREDERICK G., 1970 – Aerobic and Anaerobic Metabolism of Boar Spermatozoa in Relation to Their Motility. ***Journal of Reproduction and Fertility***, 22(1), 109-118.
23. ODET F., GABEL S.A., WILLIAMS J., LONDON R.E., GOLDBERG E., EDDY E.M., 2011 – Lactate Dehydrogenase C and Energy Metabolism in Mouse Sperm. ***Biology of Reproduction***, 85(3), 556-564.
24. O'FLAHERTY C.M., BECONI M.T., BEORLEGUI N.B., 1997 – Effect of Natural Antioxidants, Superoxide Dismutase and Hydrogen Peroxide on Capacitation of Frozen-Thawed Bull Spermatozoa. ***Andrologia***, 29(5), 269-275.
25. O'FLAHERTY C.M., BREININGER E., BEORLEGUI N.B.,BECONI M.T., 2005 – Acrosome Reaction in Bovine Spermatozoa: Role of Reactive Oxygen Species and Lactate Dehydrogenase C4. ***Biochimica et Biophysica Acta (BBA) – General Subjects***, 1726(1), 96-101.
26. O'FLAHERTY C.M., BEORLEGUI N.B., BECONI M.T., 2002 – Lactate Dehydrogenase-C4 Is Involved in Heparin- and NADH-Dependent Bovine Sperm Capacitation. ***Andrologia***, 34(2), 91-97.
27. PÉREZ M.C., PETRINELLI A., RODRIGUEZ P.C., SATORRE M.M., BREININGER E., 2019 – Comparing two simple cryopreservation methods for porcine sperm: effect on sperm quality parameters .***Revista Veterinaria***, 30(1), 23-27.
28. PHELPS M.J., LIU J., BENSON J.D., WILLOUGHBY C.E., GILMORE J.A., CRITSER J.K.,1999 – Effects of Percoll Separation, Cryoprotective Agents, and Temperature on Plasma Membrane Permeability Characteristics of Murine Spermatozoa and Their Relevance to Cryopreservation. ***Biology of Reproduction***, 61(4), 1031-1041.
29. PINTADO B., 2000 – Permeability of Boar and Bull Spermatozoa to the Nucleic Acid Stains Propidium Iodide or Hoechst 33258, or to Eosin: Accuracy in the Assessment of Cell Viability. ***Journal of Reproduction and Fertility***, 118(1), 145-152.
30. RODRÍGUEZ-GIL J.E., 2013 – Biological Aspects of the Mature Boar Spermatozoon. In: S. Bonet, I. Casas, W.V. Holt, M. Yeste (Eds.), Boar Reproduction: Fundamentals and New Biotechnological Trends, Springer, Berlin, pp 49-64.
31. RUIZ-PESINI E., DIEZ C., LAPEÑA A.C., PÉREZ-MARTOS A., MONTOYA J., ALVAREZ E., ARENAS J., LÓPEZ-PÉREZ M.J., 1998 – Correlation of Sperm Motility with Mitochondrial Enzymatic Activities. ***Clinical Chemistry***, 44(8), 1616-1620.
32. SATORRE M.M., BREININGER E., BECONI M.T., BEORLEGUI N.B., 2007 – Alpha-Tocopherol Modifies Tyrosine Phosphorylation and Capacitation-like State of Cryopreserved Porcine Sperm. ***Theriogenology,*** 68(7), 958-965.
33. SATORRE M.M., BREININGER E., BECONI M.T., BEORLEGUI N.B., 2009 – Protein tyrosine phosphorylation under capacitating conditions in porcine fresh spermatozoa and sperm cryopreserved with and without alpha tocopherol. ***Andrologia***, 41(3),184-192.
34. TRINCHERO G., PINTOS L., O'FLAHERTY C.M., BECONI M.T., 1993 – Characterization of LDHx Isoenzyme in Bovine Spermatozoa. ***Comunicaciones Biológicas***, 11, 205-215.
35. WANG W.H., ABEYDEERA L.R., FRASER L.R., NIWA K., 1995 – Functional Analysis Using Chlortetracycline Fluorescence and in Vitro Fertilization of Frozen-Thawed Ejaculated Boar Spermatozoa Incubated in a Protein-Free Chemically Defined Medium. ***Journal of Reproduction and Fertility,*** 104, 305-313.
36. YANAGIMACHI R., 1994-Mammalian fertilization. In: Knobill E.N.J (Eds), The physiology of reproduction. New York, Raven Press, pp. 109-317.