Received: 10 January 2018 | Accepted: 27 March 2018

DOI: 10.1111/rda.13197

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

Relation between respiratory activity and sperm parameters in boar spermatozoa cryopreserved with alpha-tocopherol and selected by Sephadex

MM Satorre[1,2] | E Breininger[1,2] | PD Cetica[1,2] | M Córdoba[1,2]

[1]Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal (INITRA), Universidad de Buenos Aires, Buenos Aires, Argentina

[2]Facultad de Ciencias Veterinarias, Consejo Nacional de Investigaciones Científicas y Técnicas, Investigaciones en Producción Animal (INPA), Buenos Aires, Argentina

Correspondence
Elizabeth Breininger, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Química Biológica, Buenos Aires, Argentina.
Email: ebreininger@fvet.uba.ar

Funding information
Universidad de Buenos Aires, Grant/Award Number: 20020130100281BA

Contents

Our aim was to evaluate the effect of Sephadex filtration on respiratory activity of porcine spermatozoa and its relation with quality and functional sperm parameters. Samples were evaluated regarding oxygen uptake and sperm parameters: motility, plasma and acrosome membrane integrity, capacitation and acrosome reaction induction in vitro, plasma membrane functionality, determined by the hypo-osmotic swelling test (HOST), and lipid peroxidation assessed by thiobarbituric acid assay. Sephadex filtration improved all routine quality parameters (motility, plasma and acrosome membrane integrity) and functional parameters (HOST, in vitro capacitation and true acrosome reaction levels) and produced a significant decrease in cryocapacitation and lipid peroxidation. Oxygen uptake increased in Sephadex samples (41 ± 7%) respect to single washing. Oxygen addition of carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) confirmed mitochondrial coupling in washed and Sephadex samples; showing an increase of 2.6 and 4.2 times for oxygen consumption in single washing and Sephadex ones, respectively. The increase in oxygen uptake with succinate addition with respect to basal oxygen uptake was significantly lower in Sephadex samples (63 ± 25%) than in the washed ones (183 ± 35%). Sephadex samples showed higher mitochondrial activity measured by oxygen consumption and improved quality and functional parameters. Our study recommends this protocol due to the fact that this filtration method removes dead or damaged spermatozoa allowing to obtain cryopreserved boar spermatozoa with optimized fertilizing capacity.

1 | INTRODUCTION

In sperm cells, mitochondria play a key role in generating oxidative energy and regulating sperm function. In boar spermatozoa, ATP synthesized in the mitochondria participates not only in the maintenance of sperm motility but also in the induction of capacitation and acrosome reaction (Rodriguez-Gil & Bonet, 2016). It has been suggested that mitochondrial damage during cryopreservation may be a major reason for diminished post-thaw boar sperm quality, supported by the fact that the proportion of fully functional spermatozoa that retain intact membranes and preserve mitochondrial activity after freezing-thawing is low (Dziekońska & Strzeżek, 2011).

Cryopreservation increases reactive oxygen species (ROS) levels, generating oxidative stress which results in protein damage (Davies, 1987), lipid peroxidation (Aitken, 1994) and cytoskeletal modifications (Hinshaw et al., 1986). Boar spermatozoa are especially sensitive to ROS action due to its high content of unsaturated fatty acids in plasma membrane phospholipids (Parks & Graham, 1992) and its low antioxidant capacity (Cerolini, Maldjian, Surai, & Noble, 2000). Alpha-tocopherol, an antioxidant used in cryopreservation,

 © 2018 Blackwell Verlag GmbH

improves sperm quality (Breininger, Beorlegui, O'Flaherty, & Beconi, 2005; Satorre, Breininger, Beconi, & Beorlegui, 2007) and produced a beneficial effect in the in vitro capacitation and acrosome reaction induction of boar spermatozoa (Satorre, Breininger, Beconi, & Beorlegui, 2009).

Dead or abnormal spermatozoa exert a toxic (Shannon & Curson, 1972) and/or lytic (Lindemann, Fisher, & Lipton, 1982) effect over adjacent cells, reducing the ejaculate fertility (Saacke & White, 1972). Under natural conditions, potentially fertile spermatozoa are separated from immotile ones, debris and seminal plasma in the female reproductive tract (Mortimer, 1989). In vitro, a wide range of sperm manipulation techniques are used to remove the undesirable spermatozoa, seminal plasma, cryoprotective agents and other factors (Bussalleu et al., 2008; Januskauskas, Lukoseviciute, Nagy, Johannisson, & Rodriguez-Martinez, 2005; Trentalance & Beorlegui, 2002). Sephadex filtration has been reported to improve sperm quality in bull (Cisale, Fischman, Blasi, Fernandez, & Gledhill, 2001), equine (Samper et al., 1995), canine (Mogas, Rigau, Piedrafita, Bonet, & Rodriguez-Gil, 1998) and porcine (Bussalleu et al., 2009; Satorre, Breininger, & Beconi, 2012).

The maintenance of the ability to generate oxidative energy in boar spermatozoa cryopreserved with alpha-tocopherol and selected by Sephadex generates samples with improved sperm quality and functional parameters. Thus, the aim of this study was to evaluate the effect of Sephadex filtration in boar spermatozoa cryopreserved with alpha-tocopherol on mitochondrial respiratory activity evaluated by oxygen uptake and its relation with quality (motility, membrane integrity, lipid peroxidation, acrosome integrity and cryocapacitation) and functional (membrane functionality, in vitro induction of capacitation and acrosome reaction) sperm parameters.

2 | MATERIALS AND METHODS

2.1 | Materials

Dextrose, sodium citrate, sodium bicarbonate, sodium chloride, EDTA, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and glycerol were from Merck (Darmstadt, Germany). Trypan blue, eosin yellow and nigrosin were purchased from Mallinckrodt (St. Louis, MO, USA). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 | Semen collection

Semen samples, from four crossbred boars (Pietrain × Yorkshire) of proven fertility, 1–1.5 year of age, were provided by the Porcine Productive Unit of the *Facultad de Ciencias Veterinarias (Universidad de 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 *Facultad de Ciencias Veterinarias, Universidad de Buenos Aires*. Only samples with a minimum of 70% motile and 80% viable spermatozoa were included in the study.

2.3 | Sperm cryopreservation

Boar semen was cryopreserved according to Pursel and Johnson's protocol as described by Breininger, Beorlegui, O'Flaherty et al. (2005). Briefly, each semen sample was resuspended in Beltsville F5 extender (BF5) containing 200 μg/ml alpha-tocopherol acetate to obtain a final concentration of 1×10^8 spermatozoa/ml. The contents were frozen into pellets of 0.1 ml in dry ice at −76°C and stored in liquid nitrogen at −196°C.

2.4 | Preparation of sperm suspension for selection treatment

Pellets were thawed at 38°C in Beltsville thawing solution (BTS, 2 pellets: 2 ml) and kept in a water bath for 10 min. Thawed samples were divided into two aliquots and treated with either single washing or filtration through neuter Sephadex column.

2.4.1 | Sperm single washing

Aliquots of spermatozoa-BTS suspension were centrifuged for 10 min at 300 *g*, then the supernatant was removed and the pellet was resuspended in BTS or in capacitation medium (TBMc: modified Tris-buffered medium (mTBM: NaCl 113.1 mM, KCl 3 mM, $CaCl_2$ 10 mM, Tris 20 mM, Glucose 11 mM, and sodium pyruvate mM) supplemented with sodium bicarbonate 40 mM (Satorre et al., 2009) for sperm capacitation and acrosome reaction in vitro induction (see below).

2.4.2 | Sperm filtration through neuter Sephadex column

Sephadex G-15-120 was hydrated (20%) for at least 12 hours in BTS. The column was assembled in a 10-ml disposable syringe. A small amount of glass wool was carefully placed at the bottom of the syringe body to avoid the loss of Sephadex that was deposited upon it. One millilitre of thawed sperm sample was then placed on the columns and the strangled needle removed to allow dropwise leakage. The first 5–7 drops were discarded, and aliquots of 500 μl were collected directly in BTS or centrifuged and resuspended in TBMc.

2.5 | Evaluation of sperm oxygen uptake

Oxygen consumption rate was used to evaluate mitochondrial function in intact cells. This parameter was measured polarographically at 38°C with an oxygen electrode type Clark modified 1–125/05 (Instech Laboratories Inc., Philadelphia, PA, USA). The reaction cell had 0.6 ml capacity and measured rapid changes in the cell`s oxygen consumption rate. For sperm respiration evaluation, aliquots of single washing or Sephadex-filtered samples were resuspended in TALP medium to a final concentration of 1×10^8 spermatozoa/ml in the cuvette. Basal respiration (oxygen consumption without addition of stimulator or inhibitor) was evaluated and oxygen

uptake was expressed as μl O_2/min/10^8 spermatozoa. Carbonyl-cyanide-m-chlorophenylhydrazone (CCCP; 0.42 μM) was used as a specific uncoupler of the respiratory chain. The oxygen uptake with the sequential addition of rotenone (5 μM) and sodium succinate (5 mM) to the samples was used to evaluate sperm membranes permeability to succinate (Cordoba, Mora, & Beconi, 2006).

2.6 | Evaluation of routine parameters of sperm quality

Sperm motility was evaluated three times by the same observer using an optical microscope equipped with a thermal stage at 38°C at 400× magnification (Binocular microscope XSZ 100 BN, Arcano, China). The percentage of sperm membrane integrity was determined by the supravital eosin–nigrosin stain. Staining was carried out by mixing an aliquot of sperm suspension of each treatment with the eosin (1%)–nigrosin (10%) solution (1:3 dilution) for 30 s before preparing a smear and drying on a warm plate at 38°C. At least 200 spermatozoa were counted in each sample using an optical microscope (Binocular microscope XSZ 100 BN, Arcano, China) at 400× magnification. The percentage of intact acrosomes in live cells was determined by evaluating spermatozoa stained with Trypan blue by differential-interferential contrast optical microscopy (DIC). An aliquot of the sperm suspension was incubated with an equal volume of Trypan blue (0.25%) for 15 min at 38°C, centrifuged at 600 *g* for 10 min to remove excess stain and fixed with 2% [v/v] glutaraldehyde in saline. Spermatozoa (200/sample) were assessed at 1,000× in a Jenamed-2-microscope (Carl Zeiss, Jena, Germany) (O'Flaherty, Beorlegui, & Beconi, 1999). Recovery rate of motile spermatozoa was calculated as: (total number of motile spermatozoa after treatment/total number of motile spermatozoa in non-treated sample) ×100, where total number of motile spermatozoa was calculated as: (sperm concentration x total volume of sperm sample x motility)/100. Sperm concentration was determined by haemocytometry using a Neubauer chamber.

2.7 | Evaluation of sperm capacitation level by epifluorescence chlortetracycline stain

Sperm capacitation status was evaluated through fluorescence modifications in chlortetracycline epifluorescence stain (CTC) patterns. Single washing or Sephadex-filtered samples were mixed with an equal volume of CTC solution (CTC 500 μM, NaCl 130 mM, DL-cysteine 5 mM, Tris–HCl 20 mM, pH 7.8). Glutaraldehyde (0.1%) was then added to the mixture. Slides were examined at 400× magnification under epifluorescence at 410 nm excitation filter using a Jenamed-2-fluorescence microscope (Carl Zeiss, Jena, Germany).

2.8 | Evaluation of sperm capacitation level by tyrosine phosphorylation

Tyrosine phosphorylation level was evaluated by SDS-PAGE and Western immunoblotting. Single washing or Sephadex-filtered samples were centrifuged for 5 min at 600 g and resuspended in BTS to a final concentration 4 × 10^8 spermatozoa/ml. Sodium orthovanadate (0.2 mM) was added to sperm suspension aliquots (115 μl) and then samples were centrifuged (6 min, 11,190 *g*, 4°C). The sperm pellet was resuspended in sample buffer (TRIS-HCl 62.5 mM, pH 6.8, SDS 2%, glycerol 10%) without β-mercaptoethanol and heated for 5 min at 100°C. The sperm suspension was centrifuged (30 min, 11,190 *g*, 4°C), and β-mercaptoethanol (5%) was added to the resulting supernatant. Sperm protein samples were heated for 5 min at 95°C and loaded on 12% SDS-polyacrylamide gels. Separated proteins were transferred electrophoretically to nitrocellulose membranes and labelled tyrosine phosphoproteins were visualized using a chemiluminescence detection kit (ECL, Amersham Biosciences, Piscataway, NJ, USA). Tyrosine protein phosphorylation levels were quantified in arbitrary units (AU), and boar spermatozoa, TyrP32 (pY32), were used as cryocapacitation and capacitation markers (Satorre et al., 2007, 2009).

2.9 | Evaluation of sperm lipid peroxidation

Single washing or Sephadex-filtered samples were incubated to enhance lipid peroxidation at 37°C in the presence of sodium ascorbate (0.5 mM) and ferrous sulphate (0.11 mM) for 2 hr. The lipid peroxidation was determined by measuring the level of substances reacting with 2-thiobarbituric acid (TBARS) using a Shimadzu F 1501 spectrofluorometer (Shimadzu Corporation, Kyoto, Japan; Beorlegui, Cetica, Trinchero, Cordoba, and Beconi (1997). TBARS levels are expressed as nmol TBARS/10^8 spermatozoa.

2.10 | Evaluation of sperm membrane functionality (Hypo-osmotic swelling test—HOST)

Single washing or Sephadex-filtered samples (50 μl) were mixed with 500 μl of hypo-osmotic solution (sodium citrate 0.49%, fructose 0.9%, 100 mOsm/kg) and incubated at 38°C for 60 min. Two hundred spermatozoa were counted by differential-interferential contrast (DIC) microscopy in a Jenamed- 2-microscope (Carl Zeiss, Jena, Germany; Zhao et al., 2009), and spermatozoa with functional membranes presented a swelling in the tail membrane.

2.11 | In vitro sperm capacitation and acrosome reaction induction

Single washing or Sephadex-filtered samples were incubated in TBMc for 30 min at 38°C under 5% CO_2 in humidified air. In vitro capacitation was evaluated by CTC and tyrosine phosphorylation as described above. The percentages of capacitated spermatozoa were obtained by subtracting the values obtained at zero time of incubation, to rule out cells destabilized during the freezing-thawing process. The ability of capacitated spermatozoa to undergo acrosome reaction was assessed as follows: capacitated samples were incubated with follicular fluid (FF) (30% v/v) at 38°C under 5% CO_2 in humidified air for 15 min (Breininger, Beorlegui, & Beconi, 2005).

TABLE 1 Oxygen consumption of cryopreserved boar spermatozoa with alpha-tocopherol selected by Sephadex

	Treatment	
O_2 consumption	SW	SF
Basal (µl O_2/min/10^8 sp)	$2.22 \times 10^{-08} \pm 0.65 \times 10^{-08}$ a	$3.90 \times 10^{-08} \pm 0.68 \times 10^{-08}$ b
CCCP (µl O_2/min/10^8 sp)	$7.95 \times 10^{-08} \pm 0.28 \times 10^{-08}$ a,*	$16.2 \times 10^{-08} \pm 0.32 \times 10^{-08}$ b,*
Rotenone/Succinate (µl O_2/min/10^8 sp)	$4.88 \times 10^{-08} \pm 0.95 \times 10^{-08}$ a,**	$5.90 \times 10^{-08} \pm 0.85 \times 10^{-08}$ a,**

SW, single washing, SF, Sephadex filtration.
Data are expressed as means ± SD of 5 replicates. For each parameter of O_2 consumption: superscript letters (a–b) indicate significant differences between treatments (SW or SF) ($p < .05$). For the same treatment (SW or SF) *, ** indicate significant differences between oxygen consumption with CCCP or rotenone/succinate values with respect to basal respiration ($p < .05$).

True acrosome reaction (TAR) was evaluated by the combined technique of DIC and Trypan Blue stain as described previously. TAR value at zero time was subtracted to rule out spontaneous acrosome reaction.

2.12 | Statistical analysis

Results are expressed as mean ± SD. A pool of cryopreserved spermatozoa (two ejaculates from four different boars) was used for each experiment, and five replicates were performed. Analysis of covariance was previously performed to ensure that the differences in sperm quality among samples did not interfere in the results obtained. The evaluation of the effect of stimulators or inhibitors of mitochondrial activity on oxygen consumption with respect to basal values was performed by a paired *t* test. The effect of the treatments in the different experiences was analysed by a two-sample *t* test. The association between B-CTC pattern and arbitrary units of tyrosine protein phosphorylation was analysed using Pearson's correlation coefficients. A value of $p < .05$ was considered as statistically significant. All statistical tests were performed with InfoStat (*Universidad de Córdoba, Córdoba, Argentina*, see http://www.infostat.com.ar/).

3 | RESULTS

Oxygen consumption rate in Sephadex-filtered (SF) samples increased 41 ± 7% with respect to single washing (SW) samples ($p < .05$). Oxygen uptake with CCCP confirmed mitochondrial coupling in SW and SF samples, finding that oxygen consumption was significantly higher in both samples respect to those without the uncoupler ($p < .05$). An increase of 2.6 and 4.2 times for oxygen consumption was observed in SW and SF samples, respectively (Table 1). The increase in oxygen consumption with succinate (in the presence of rotenone) in respect to basal oxygen consumption was significantly lower in SF samples (63 ± 25%) than in SW ones (183 ± 35%).

Sephadex improved all the routine parameters of sperm quality evaluated. ($p < .05$). The concentration after treatments was $(4.56 \pm 0.64) \times 10^7$ spermatozoa and $(2.81 \pm 0.69) \times 10^7$ spermatozoa, for SW and SF samples, respectively. The recovery rate was statistically different: 84 ± 2 and 61 ± 7 for SW and SF samples, respectively. A significant decrease in cryocapacitation levels, measured by CTC (pattern B), and tyrosine phosphorylation (pY32) was observed in SF samples ($p < .05$) being these results highly correlated ($r = .86$, $p < .05$). A significant decrease in lipid peroxidation levels, measured by TBARS, compared with SW ones ($p < .05$, Table 2).

TABLE 2 Sperm quality parameters in cryopreserved boar spermatozoa with alpha-tocopherol selected by Sephadex

	Treatment	
Parameter	SW	SF
Motility (%)	38.5 ± 5.2^a	62.6 ± 8.5^b
Membrane integrity (%)	39.3 ± 4.9^a	60.2 ± 5.9^b
Acrosome integrity (%)	39.6 ± 2.7^a	50.4 ± 3.7^b
Cryocapacitation (B-CTC, %)	18.1 ± 1.4^a	10.9 ± 1.7^b
Cryocapacitation pY32 (A.U.)	1.4 ± 0.2^a	1.1 ± 0.1^b
Lipid peroxidation (nm TBARS/10^8 sp).	21.7 ± 2.0^a	12.0 ± 0.6^b

SW, single washing; SF, Sephadex filtration.
Data are expressed as means ± SD of 5 replicates. For each parameter: superscript letters (a–b) indicate significant differences between treatments (SW or SF) ($p < .05$).

All functional parameters were improved by Sephadex filtration ($p < .05$). An increase in the percentage of capacitated spermatozoa (evaluated by B-CTC pattern and pY32) was observed in SF samples with respect to SW ones ($p < .05$), being these parameters highly correlated ($r = .88$, $p < .05$). The percentage of capacitated spermatozoa that underwent true acrosome reaction after induction was higher in SF samples than in SW ones ($p < .05$, Table 3).

4 | DISCUSSION

Increased permeability of plasma and mitochondrial membranes constitute a major event in cell damage and dysfunction observed during sperm cryopreservation (Zhu & Liu, 2000). Mitochondria,

TABLE 3 Sperm functional parameters in cryopreserved boar spermatozoa with alpha-tocopherol selected by Sephadex

Parameter	Treatment	
	SW	SF
Membrane functionality, HOST (%)	31.5 ± 3.0[a]	42.5 ± 5.5[b]
In vitro capacitation, B-CTC (%)	20.7 ± 2.6[a]	27.9 ± 4.4[b]
In vitro capacitation, pY32 (A.U)	1.3 ± 0.2[a]	1.6 ± 0.2[b]
In vitro acrosomal reaction AT-DIC (%).	14.6 ± 2.4[a]	24.8 ± 3.5[b]

SW, single washing, SF, Sephadex filtration.
Data are expressed as means ± SD of five replicates. For each parameter: superscript letters (a–b) indicate significant differences between treatments (SW or SF) ($p < .05$).

which contain the enzymatic machinery to generate oxidative energy, seem to be the most sensitive of sperm structures to freezing and thawing (Cummins, Jequier, & Kan, 1994). Cryopreservation produces changes in mitochondrial function that may be reflected in sperm motility (Ericsson, Garner, Thomas, Downing, & Marshall, 1993). In porcine species, antioxidant supplementation during cryopreservation has resulted in positive effects on sperm motility and maintenance of mitochondrial membrane potential after thawing (Peña, Johannisson, Wallgren, & Rodriguez Martinez, 2003). In this study, boar sperm samples cryopreserved with alpha-tocopherol and selected by Sephadex filtration presented higher oxygen consumption and increased sperm motility, along with the improvement in the structural and functional integrity of their membrane, suggesting the possibility of selecting sperm with higher oxidative activity.

Although Sephadex filtration is a technique used in many species for sperm selection, the mechanism by which Sephadex retains dead or damaged spermatozoa has not been elucidated yet. Only functional spermatozoa are able to cross Sephadex matrix (Brito, Barth, Bilodeau-Goeseel, Panich, & Kastelic, 2003) due to a complex aerodynamic phenomenon that involves the countercurrent orientation of the sperm with progressive motility (Cisale et al., 2001). According to that, the changes produced in the plasmatic and/or acrosomal membranes during cryopreservation would modify the hydrodynamic profile of the spermatozoa or its progressive motility favouring its retention by Sephadex (Fernandez, Cisale, & Aisen, 1985; Harayama, 2003). In our research, we demonstrated that Sephadex selection of cryopreserved samples with alpha-tocopherol contributed to obtain samples with preserved mitochondrial function that is required for different sperm processes. According to this, samples selected by Sephadex would have a greater fertilizing potential due to its improved motility, plasma membrane integrity and function, acrosomal integrity, reduced cryocapacitation and lipid peroxidation levels. These samples could be used for laboratory purposes (as in vitro fertilization protocols) or in porcine production using alternative methods of artificial insemination as intrauterine and deep intrauterine techniques, as these techniques utilize a low number of spermatozoa per insemination doses (Yeste, Rodriguez-Gil, & Bonet, 2017).

One method to evaluate mitochondrial function is the oxygen uptake polarographic assay which determines active respiring mitochondria. This method allows the evaluation of not only the total respiratory capacity but also the proper function of the multienzymatic complexes of the mitochondrial chain (Ferramosca, Provenzano, Coppola, & Zara, 2012). Uncouplers are chemical compounds which dissociate electron transfer from oxidative phosphorylation. In particular, CCCP is a weak acid with hydrophobic properties which allows it to diffuse across mitochondrial membranes. As the proton gradient dissipates, ATP production is prevented, but respiration continues. In compensation, the electron transport speeds up and consequently oxygen consumption increases (Nelson & Cox, 2014). Although it is known that cryopreservation alters plasma membrane (Zhu & Liu, 2000) and mitochondrial integrity (Nishizono, Shioda, Takeo, Irie, & Nakagata, 2004), we demonstrated that mitochondria are capable of retaining respiratory capacity, as indicated by the increase in respiration induced by the uncoupler. Similar results were observed in cryopreserved bull (Cordoba et al., 2006) and horse (Schober, Aurich, Nohl, & Gille, 2007) spermatozoa.

Rotenone is a strong inhibitor of complex I of the mitochondrial electron transport chain, but allows the transport of electrons through the complex II, using succinate as oxidative substrate (Nelson & Cox, 2014). A high oxygen consumption with succinate has been previously demonstrated in cryopreserved bovine (Beconi, Beorlegui, & Diz de Otamendi, 1986) and horse (Agüero et al., 1995) spermatozoa. In accordance, an increase in succinate permeability in cryopreserved boar sperm samples was observed. Unlike fresh boar semen, no oxygen consumption was observed in the presence of rotenone/succinate (Satorre, Beconi, Breininger, & Cordoba, 2014). The rotenone/succinate respiration ratio with respect to basal values in single washing samples was higher than Sephadex treated ones. This lower relation could be due to the increase in mitochondrial and plasma membranes integrity in Sephadex samples which implies that less succinate is incorporated as a mitochondrial oxidative substrate.

The negative association between sperm quality parameters and TBARS production has suggested that lipid peroxidation alters mechanisms or structures that lead to motility decrease, as observed in bovine (Beorlegui et al., 1997) and boar (Breininger, Beorlegui, O'Flaherty et al., 2005) cryopreserved semen. The ability of alpha-tocopherol to inhibit lipid peroxidation in boar semen is well known (Cerolini et al., 2000). The level of respiratory activity observed in Sephadex samples with respect to washed ones would be related to the decrease in lipid peroxidation and therefore a higher membrane integrity.

Thus, Sephadex-selected samples showed higher mitochondrial activity measured by oxygen uptake, this is reflected in improved quality and functional parameters. Several studies confirm that sperm motility is associated with mitochondrial functional status in human (Ferramosca et al., 2012), horse (Love et al., 2003), rat (Gravance, Garner, Miller, & Berger, 2001), pig (Spinaci et al., 2005) and ram (Martinez-Pastor et al., 2004). In this way, we infer that mitochondrial function and membrane architecture may be the most important indicator to explain the fertility decrease in cryopreserved

semen, in accordance with Cummins et al. (1994). Our study recommends this protocol to optimize the fertilizing capacity of cryopreserved boar spermatozoa as this filtration method removes dead or damaged spermatozoa.

ACKNOWLEDGEMENTS

This research was supported by the *Universidad de Buenos Aires*, (UBACyT 20020130100281BA) "*Estudio de parámetros bioquímicos y funcionales de espermatozoides de especies vinculadas con producción animal y la conservación de la biodiversidad. Directora Mariana Córdoba.*" We are grateful to ASTRA for ultrapure water and Claudio García for the collection of the samples.

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

María Mercedes Satorre performed the experiences to evaluate sperm parameters and oxygen uptake, and participated in the analysis and interpretation of data. Elizabeth Breininger contributed to perform the experiences to evaluate sperm parameters, performed the analysis and interpretation of data, and drafted the manuscript. Pablo Cetica contributed to the analysis and interpretation of data, and drafted the article. Mariana Córdoba contributed to perform the experiences to evaluate oxygen uptake, participates in the analysis and interpretation of data and drafted the manuscript.

ORCID

E Breininger http://orcid.org/0000-0001-7185-0190

REFERENCES

Agüero, A., Miragaya, M. H., Mora, N. G., Chaves, M. G., Neild, D. M., & Beconi, M. T. (1995). Effect of vitamin E addition on equine sperm preservation. *Comunicaciones Biologicas*, *13*(4), 343–356.

Aitken, R. J. (1994). A free radical theory of male infertility. *Reproduction, Fertility, and Development*, *6*(1), 19–23. https://doi.org/10.1071/RD9940019

Beconi, M. T., Beorlegui, N. B., & Diz de Otamendi, M. (1986). Capacidad fosforilante y permeabilidad al succinato en semen bovino congelado. *Revue Médicine Vétérinaire*, *67*, 120–126.

Beorlegui, N., Cetica, P., Trinchero, G., Cordoba, M., & Beconi, M. (1997). Comparative study of functional and biochemical parameters in frozen bovine sperm. *Andrologia*, *29*(1), 37–42.

Breininger, E., Beorlegui, N. B., & Beconi, M. T. (2005). Efecto diferencial del fluido folicular y la zona pellucida en la reacción acrosomal en semen porcino criopreservado [Differential effect of follicular fluid and the zona pellucida on the acrosomal reaction in cryopreserved porcine semen]. Poster session presented at the meeting of the Instituto de Reproducción Animal Córdoba, Argentina.

Breininger, E., Beorlegui, N. B., O'Flaherty, C. M., & Beconi, M. T. (2005). Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. *Theriogenology*, *63*(8), 2126–2135. https://doi.org/10.1016/j.theriogenology.2004.08.016

Brito, L., Barth, A., Bilodeau-Goeseel, S., Panich, P., & Kastelic, J. (2003). Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. *Theriogenology*, *60*, 1539–1551. https://doi.org/10.1016/S0093-691X(03)00174-2

Bussalleu, E., Pinart, E., Rivera, M. M., Arias, X., Briz, M., Sancho, S., … Bonet, S. (2008). Effects of filtration of semen doses from subfertile boars through neuter Sephadex columns. *Reproduction in Domestic Animals*, *43*(1), 48–52.

Bussalleu, E., Pinart, E., Rivera, M.M., Briz, M., Sancho, S., Yeste, M., … Bonet, S. (2009). Effects of matrix filtration of low-quality boar semen doses on sperm quality. *Reproduction in Domestic Animals*, *44*(3), 499–503. https://doi.org/10.1111/j.1439-0531.2008.01221.x

Cerolini, S., Maldjian, A., Surai, P., & Noble, R. (2000). Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim. Reproductive Sciences*, *58*(1–2), 99–111. https://doi.org/10.1016/S0378-4320(99)00035-4

Cisale, H. O., Fischman, M. L., Blasi, C. D., Fernandez, H. A., & Gledhill, B. L. (2001). Enrichment of high-quality spermatozoa in bovine semen: Relative effectiveness of three filtration matrixes. *Andrologia*, *33*(3), 143–150. https://doi.org/10.1046/j.1439-0272.2001.00413.x

Cordoba, M., Mora, N., & Beconi, M. T. (2006). Respiratory burst and NAD(P)H oxidase activity are involved in capacitation of cryopreserved bovine spermatozoa. *Theriogenology*, *65*(4), 882–892. https://doi.org/10.1016/j.theriogenology.2005.06.015

Cummins, J. M., Jequier, A. M., & Kan, R. (1994). Molecular biology of human male infertility: Links with aging, mitochondrial genetics, and oxidative stress? *Molecular Reproduction and Development*, *37*(3), 345–362. https://doi.org/10.1002/(ISSN)1098-2795

Davies, J. K. (1987). Protein damage and degradation by oxygen radicals I. General aspects. *J. Biological Chemistry*, *262*(9895–9901).

Dziekońska, A., & Strzeżek, J. (2011). Boar variability affects sperm metabolism activity in liquid stored semen at 5°C. *Pol. Journal of Veterinary Science*, *14*(1), 21–27.

Ericsson, S. A., Garner, D. L., Thomas, C. A., Downing, T. W., & Marshall, C. E. (1993). Interrelationships among fluorometric analyses of spermatozoal function, classical semen quality parameters and the fertility of frozen-thawed bovine spermatozoa. *Theriogenology*, *39*(5), 1009–1024. https://doi.org/10.1016/0093-691X(93)90002-M

Fernandez, H. A., Cisale, H. O., & Aisen, E. G. (1985). Mecanismo de mejoramiento de semen bovino por filtración a través de Sephadex. *Vet Argentina*, *2*, 144–148.

Ferramosca, A., Provenzano, S. P., Coppola, L., & Zara, V. (2012). Mitochondrial respiratory efficiency is positively correlated with human sperm motility. *Urology*, *79*(4), 809–814. https://doi.org/10.1016/j.urology.2011.12.042

Gravance, C. G., Garner, D. L., Miller, M. G., & Berger, T. (2001). Fluorescent probes and flow cytometry to assess rat sperm integrity and mitochondrial function. *Reproductive Toxicology*, *15*(1), 5–10. https://doi.org/S0890623800001131

Harayama, H. (2003). Viability and protein phosphorylation patterns of boar spermatozoa agglutinated by treatment with a cell-permeable cyclic adenosine 3′, 5′- monophosphate analog. *Journal of Andrology*, *24*, 831–842. https://doi.org/10.1002/j.1939-4640.2003.tb03134.x

Hinshaw, D. B., Sklar, L. A., Bohl, B., Schraufstatter, I. U., Hyslop, P. A., Rossi, M. W., … Cochrane, C. G. (1986). Cytoskeletal and morphologic impact of cellular oxidant injury. *American Journal of Pathology*, *123*(3), 454–464.

Januskauskas, A., Lukoseviciute, K., Nagy, S., Johannisson, A., & Rodriguez-Martinez, H. (2005). Assessment of the efficacy of Sephadex G-15 filtration of bovine spermatozoa for

cryopreservation. *Theriogenology*, *63*(1), 160–178. https://doi.org/10.1016/j.theriogenology.2004.04.002

Lindemann, C. B., Fisher, M., & Lipton, M. (1982). A comparative study of the effects of freezing and frozen storage on intact and demembranated bull spermatozoa. *Cryobiology*, *19*(1), 20–28. https://doi.org/10.1016/0011-2240(82)90121-3

Love, C. C., Thompson, J. A., Brinsko, S. P., Rigby, S. L., Blanchard, T. L., Lowry, V. K., & Varner, D. D. (2003). Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. *Theriogenology*, *60*(6), 1127–1138. https://doi.org/S0093691X03001225

Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., & Rodriguez-Martinez, H. (2004). Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen . *Animal Reproductive Sciences*, *84*(1–2), 121–133. https://doi.org/10.1016/j.anireprosci.2003.12.006

Mogas, T., Rigau, T., Piedrafita, J., Bonet, S., & Rodriguez-Gil, J. E. (1998). Effect of column filtration upon the quality parameters of fresh dog semen. *Theriogenology*, *50*(8), 1171–1189. https://doi.org/10.1016/S0093-691X(98)00218-0

Mortimer, D. (1989). Sperm transport in the human female reproductive tract. In C. A. Finn (Ed.), *Oxford reviews of reproductive biology* (Vol *5*). Oxford, UK: Oxford University Press.

Nelson, D.L., & Cox, M.M. (2014). *Lehninger: Principles of biochemistry* (5th edn). New York, NY: W.H. Freeman.

Nishizono, H., Shioda, M., Takeo, T., Irie, T., & Nakagata, N. (2004). Decrease of fertilizing ability of mouse spermatozoa after freezing and thawing is related to cellular injury. *Biology of Reproduction*, *71*(3), 973–978. https://doi.org/10.1095/biolreprod.103.024422

O'Flaherty, C. M., Beorlegui, N. B., & Beconi, M. T. (1999). Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. *Theriogenology*, *52*(2), 289–301. https://doi.org/10.1016/S0093-691X(99)00129-6

Parks, J. E., & Graham, J. K. (1992). Effects of cryopreservation procedures on sperm membranes. *Theriogenology*, *38*(2), 209–222. https://doi.org/10.1016/0093-691X(92)90231-F

Peña, F. J., Johannisson, A., Wallgren, M., & Rodriguez Martinez, H. (2003). Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Anim. Reproductive Sciences*, *78*(1–2), 85–98.

Rodriguez-Gil, J. E., & Bonet, S. (2016). Current knowledge on boar sperm metabolism: Comparison with other mammalian species. *Theriogenology*, *85*(1), 4–11. https://doi.org/10.1016/j.theriogenology.2015.05.005

Saacke, R.G., & White, J.M. (1972). Semen quality tests and their relationship to fertility. Proceeding of the 4th Technical Conference on AI and Reprod., NAAB, USA, pp. 22–27.

Samper, J. C., Hamilton, D. W., Pryor, J. L., Loseth, K. J., Troedsson, M. H. T., & Crabo, B. G. (1995). Mechanism of Sephadex trapping of capacitated stallion spermatozoa. *Biology of Reproduction*, *1*, 729–737.

Satorre, M. M., Beconi, M. T., Breininger, E., & Cordoba, M. (2014). Mitochondrial activity in fresh and frozen with or without α-tocopherol boar semen. *INVET*, *16*, 142.

Satorre, M. M., Breininger, E., & Beconi, M. T. (2012). Cryopreservation with alpha-tocopherol and Sephadex filtration improved the quality of boar sperm. *Theriogenology*, *78*(7), 1548–1556. https://doi.org/10.1016/j.theriogenology.2012.06.023

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. https://doi.org/10.1016/j.theriogenology.2007.06.021

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. https://doi.org/10.1111/j.1439-0272.2009.00915.x

Schober, D., Aurich, C., Nohl, H., & Gille, L. (2007). Influence of cryopreservation on mitochondrial functions in equine spermatozoa. *Theriogenology*, *68*(5), 745–754. https://doi.org/10.1016/j.theriogenology.2007.06.004

Shannon, P., & Curson, B. (1972). Toxic effect and action of dead sperm on diluted bovine semen. *Journal of Dairy Science*, *55*(5), 614–620. https://doi.org/10.3168/jds.S0022-0302(72)85544-9

Spinaci, M., De Ambrogi, M., Volpe, S., Galeati, G., Tamanini, C., & Seren, E. (2005). Effect of staining and sorting on boar sperm membrane integrity, mitochondrial activity and in vitro blastocyst development. *Theriogenology*, *64*(1), 191–201. https://doi.org/S0093-691X(04)00400-5

Trentalance, G. M., & Beorlegui, N. B. (2002). Sperm evaluation in cryopreserved bovine semen recovered by two selection methods. *Andrologia*, *34*(6), 397–403. https://doi.org/10.1046/j.1439-0272.2002.00525.x

Yeste, M., Rodriguez-Gil, J. E., & Bonet, S. (2017). Artificial insemination with frozen-thawed boar sperm. *Molecular Reproduction and Development*, *84*(9), 802–813. https://doi.org/10.1002/mrd.22840

Zhao, H. W., Li, Q. W., Ning, G. Z., Han, Z. S., Jiang, Z. L., & Duan, Y. F. (2009). Rhodiola sacra aqueous extract (RSAE) improves biochemical and sperm characteristics in cryopreserved boar semen. *Theriogenology*, *71*(5), 849–857. https://doi.org/10.1016/j.theriogenology.2008.10.006

Zhu, W., & Liu, X. G. (2000). Cryodamage to plasma membrane integrity in head and tail region human sperm. *Journal of Andrology*, *2*, 135–138.

How to cite this article: Satorre MM, Breininger E, Cetica PD, Córdoba M. Relation between respiratory activity and sperm parameters in boar spermatozoa cryopreserved with alpha-tocopherol and selected by Sephadex. *Reprod Dom Anim*. 2018;53:979–985. https://doi.org/10.1111/rda.13197