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Is boar sperm freezability more intrinsically linked to spermatozoa than to the surrounding seminal plasma?

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Abstract

This study aimed to elucidate the effect of SP from post-SRF on boar sperm freezability and, in addition, to determine the relevance of sperm itself to sustain cryopreservation, regardless of the SP surrounding them. Twelve ejaculates from three boars were manually collected in fractions/portions, P1: the first 10 mL of the SRF, P2: the rest of the SRF and the post-SRF. Immediately, samples were centrifuged to separate spermatozoa from the surrounding SP. Spermatozoa from P1 and P2 were then incubated with its own SP or that from post-SRF, diluted in BTS (1:1, v/v) at 17 °C overnight before being frozen in 0.5 mL straws using a standard protocol. Sperm motility (total and progressive) deteriorated ($P < 0.05$) when P1- or P2-sperm when incubated overnight in SP from post-SRF, while sperm viability differed between P1 and P2 ($P < 0.05$) regardless of the SP they were incubated in. Post-thaw sperm quality and functionality differed between P1 and P2, regardless of the SP used for overnight pre-freezing incubation. Post-thaw motility ($P < 0.05$) and viability ($P < 0.01$), as well as plasma membrane fluidity ($P < 0.05$) or lipid peroxidation values ($P < 0.01$) were best in P1 sperm compared to those of P2. The protein profile of sperm from P1 and P2, analyzed by 2D-PAGE, showed qualitative differences, which suggest that sperm rather than SP would explain differences in sperm freezability between ejaculate fractions/portions. Use of P1 fraction spermatozoa seems thus optimal for cryopreservation.

Keywords: sperm cryopreservation; seminal plasma; ejaculate portions; sperm protein profile; boar

1. Introduction

Cryopreservation is as yet the most useful tool to long-time store pig semen, and its commercial implementation in artificial insemination (AI) programs would suppose numerous advantages for the porcine industry (reviewed by Yeste et al., 2017). However, to date, frozen-thawed spermatozoa are scarcely used in commercial AI-programs due, mainly, to the lower fertility of the frozen-thawed semen compared to the use of liquid-stored slightly cooled semen (reviewed by Knox, 2015). The high sensitivity of boar spermatozoa to cryopreservation is the main reason explaining the poorer fertility results of frozen-thawed semen (Yeste et al., 2017).

Current knowledge that the seminal plasma (SP) is much more than a nutrient medium in which spermatozoa are transported during ejaculation and sperm transport in the female (Juyena and Stelletta, 2012), has propitiated that many researchers have focused on the role of SP on the capability of boar sperm for sustaining cryopreservation (Yeste, 2015, 2016). One of the most striking results were the differences in sperm cryosensitivity among ejaculate fractions (Saravia et al., 2009), with sperm rich ejaculate fraction (SRF) sustaining better cryoresistance than those from the entire ejaculate (including SRF and post-SRF). These results led to the suggestion that the SP from the post-SRF impairs freezability of boar sperm (Saravia et al., 2009; Alkmin et al., 2014). This finding has special relevance today, as most pig AI-centers are moving, for hygiene and labor cost, from using manual and selective ejaculate collection (most often collecting only the SRF fraction), to semi-automatic methods where the entire ejaculate is collected in one vial, without any distinction of fractions (Aneas et al., 2008). A recent study showed that SP-antioxidants contribute to explain differences in sperm freezability between SRF and entire ejaculate (Li et al., 2018). However, whether it is the SP of post-SRF that impairs freezability, remains unclear.

Recent studies have identified quantitative differences in specific SP (Vilagran et al., 2015) and sperm (Chen et al., 2014; Vilagran et al., 2013 and 2014; Guimarães et al., 2017) proteins between sires, with clear differences in sperm freezability. These studies highlighted that both the protein composition of SP but also that of sperm, could explain differences between boars regarding the ability of their sperm to sustain cryopreservation.

To aid explaining the reasons for cryosurvival differences between SRF, either the first 10 mL or the rest of SRF, with the entire ejaculate, the present study was designed to (i) clarify the effect of SP from post-SRF on the freezability of boar spermatozoa and (ii) evaluate whether the SP is the only responsible for such eventual putative differences. Considering that an incubation period of sperm with SP for up to 24 h is recommended for best cryosurvival (Eriksson et al., 2001), cleansed ejaculated spermatozoa (freed from SP immediately after ejaculation) from both the first 10 mL of SRF and the rest of SRF were incubated overnight before freezing with its own SP or with SP from the post-SRF. In addition, sperm protein profiling (2D-PAGE) among ejaculate fractions was evaluated.

2. Material and methods

2.1. Reagents and media

Unless otherwise stated, all chemicals used in the experiments were of analytical grade purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The media were prepared under sterile conditions in a laminar flow hood (MicroH; Telstar, Terrasa, Spain). The basic medium used to extend semen was Beltsville Thawing Solution (BTS: 205 mM glucose, 20.39 mM $\text{Na}_3\text{-C}_6\text{H}_5\text{O}_7$, 10.0 mM KCl, 15.01 mM NaHCO_3 , 3.36 mM EDTA; pH 7.2 and 290–300 mOsmol/kg) supplemented with 50 mg/mL kanamycin sulfate. EDTA-free phosphate-buffered saline (PBS: 139 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.1 mM

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; with 0.058 g/L penicillin G and 0.05 g/L streptomycin sulphate; pH 6.8 and 280–300 mOsmol/kg) was used to dilute fluorochromes and extend sperm samples when needed. Sperm were frozen using a basic freezing extender containing 80% (v/v) Tris-citric acid-glucose extender (111 mM Trizma Base, 31.4 mM monohydrate citric acid, 185 mM glucose) and 20% (v/v) egg yolk, supplemented with 100 $\mu\text{g}/\text{mL}$ kanamycin sulfate (pH 7.2 and 295–300 mOsmol/kg).

2.2. Boars and ejaculates

All procedures that involved animals were performed following international guidelines (Directive 2010/63/EU) and were approved in advance by the Bioethics Committee of Murcia University (research code: 639/2012).

Three healthy, sexually mature and fertility-proven boars (two Large White and one Landrace) were used as semen providers in this study. All boars belonged to Topigs Norsvin España (Madrid, Spain) and were housed in a Spanish AI-center located in Murcia. The boars were housed in individual pens under controlled temperature (15–25 °C) and 16 h of natural/artificial daylight with free access to water and fed with a commercial feedstuff to meet the nutritional requirements of adult boars subjected to regular ejaculate collection (two ejaculates per week). A total of 12 ejaculates (four per boar) were fractionally collected by using the gloved-hand method. The portions collected separately were the first 10 ml of the SRF (P1), the rest of the SRF (P2) and the post-SRF.

2.3. Preparation of sperm samples and seminal plasma processing

Immediately after ejaculate collection, P1 and P2 portions that fulfilled the minimal requirements of sperm quality (more than 75% of sperm motility and 85% sperm with normal

morphology), were split into three aliquots of 3 mL, which were centrifuged at 800 x g during 10 min (Rotofix 32A; Hettich Zentrifugen, Tuttlingen, Germany). The supernatant SP was again centrifuged (1,500 x g, 10 min) to obtain sperm free-SP, confirmed after light microscopy examination. Sperm pellets of one aliquot of P1 and P2 were extended with SP of its own fraction, whereas those of the other aliquot were extended with SP from post-SRF until reaching the original volume of 3 mL. Thereby, four semen samples per ejaculate were obtained, specifically (1) sperm from P1 + SP from P1, (2) sperm from P1 + SP from post-SRF, (3) sperm from P2 + SP from P2, and (4) sperm from P2 + SP from post-SRF. The resulting 48 semen samples were further extended 1:1 (v/v) with BTS, put into tubes trying to avoid air bubbles in the tubes. The sperm pellets of the third aliquot were washed twice with PBS (1,500 x g, 10 min) and the resulting pellets were further extended with PBS until reaching the original 3-mL-volume. All samples were thereafter promptly transported (< 2 h) in thermal containers to the Andrology Laboratory of Veterinary Teaching Hospital (VTH) of University of Murcia.

2.4. Sperm cryopreservation

On the next morning, before freezing, sperm quality (sperm concentration, motility, viability and morphology) of each prepared semen sample was evaluated. Thereafter, the samples were centrifuged (Megafuge 1.0 R, Heraeus, Hanau, Germany) at 2,400 x g for 3 min, and the sperm pellets frozen using the 0.5 mL-straw freezing procedure described by Alkmin et al. (2014). Briefly, sperm pellets were re-extended in freezing extender (FE) to 1.5×10^9 cells/mL. After cooling to 5°C for 150 min, sperm cells were re-extended with FE-glycerol-Equex extender [89.5% FE+1.5% Equex STM (v/v) (Nova Chemical Sales, Scituate, MA, USA) + 9% glycerol (v/v); pH: 6.2; 1,700-1,730 mOsmol/kg] to a final concentration

of 1.0×10^9 cells/mL. Immediately thereafter, the spermatozoa were packed into 0.5 mL polyvinyl chloride (PVC) French straws (Minitüb, Tiefenbach, Germany) and equilibrated at 3 cm above liquid nitrogen (LN_2) for 20 min. Then the straws were plunged into LN_2 and transferred to a LN_2 tank for at least one-week storage. Thawing was then performed in a circulating water bath at 37 °C for 20 s and then the straw content was re-extended in BTS (1:1, v/v) and kept in a dark chamber at 37°C during 150 min.

2.5. Assessment of sperm quality and functionality

Sperm quality in terms of objective total and progressive motility and viability were evaluated at 17°C after overnight storage prior to freezing. After thawing, the same parameters of sperm quality and sperm functionality, in terms of plasma membrane fluidity, production of intracellular ROS, proportions of cells undergoing early apoptosis and lipid peroxidation, were evaluated at 30 and 150 min post-thawing.

2.5.1. Total and progressive motility

The motility of the spermatozoa was objectively evaluated by using a computer-assisted analysis system (ISAS; Proiser R+D, Paterna, Spain). Briefly, the sperm samples were re-suspended in BTS to a concentration of $20\text{--}30 \times 10^6$ cells/mL. For each evaluation, 5 μL of each sperm sample were placed in a pre-warmed Makler counting chamber (38 °C), and 4 to 5 fields were visualized to analyse a minimum of 400 sperm per sample. Before the track sequence was analysed, the trajectory of each spermatozoa identified and recorded in each field were assessed visually to eliminate possible debris and to decrease the risk of including unclear tracks in the analysis. The recorded sperm motility variables were the overall

percentage of total motile sperm (average path velocity $\geq 20 \mu\text{m/s}$) and percentage of sperm depicting rapid and progressive movement (straight line velocity $\geq 40 \mu\text{m/s}$).

2.5.2. Viability

Sperm viability was evaluated by simultaneous cytometric assessment of the plasma and acrosome membrane integrity by using a triple-fluorescence procedure. Briefly, 100 μL of each sperm sample (30×10^6 cells/mL in BTS) were transferred to culture tubes containing 3 μL Hoechst 33342 (H-42; 0.05 mg/mL in PBS), 2 μL propidium iodide (PI, 0.5 mg/mL in PBS) and 2 μL fluorescein-conjugated peanut agglutinin (PNA-FITC, 200 $\mu\text{g/mL}$ in PBS). The samples were mixed and incubated at 38 °C in the dark for 10 min. Before analysis by flow cytometry, 400 μL PBS were added to each sample. A total of 10,000 sperm were recorded. Sperm H-42 positive/PI and PNA-FITC negative were recorded as viable.

2.5.3. Plasma membrane fluidity

The fluidity of the sperm plasma membrane was assessed by staining the sperm samples with H-42, Merocyanine 540 (M-540) and Yo-Pro-1 (Molecular Probes Europe BV, Leiden, The Netherlands). Aliquots of 50 μL (30×10^6 spermatozoa/mL) were extended in 950 μL of PBS containing 2.5 μL of H-42 (0.05 mg/mL in PBS) and 10 μL of Yo-Pro-1 (2.5 μM in DMSO) and incubated at 38 °C for 8 min in the dark. Then, 26 μL of M-540 (0.1 mM in DMSO) was added to each sample, and the samples incubated for another 2 min under the same conditions before flow cytometric analysis. A total of 10,000 sperm were recorded. Sperm H-42 positive, Yo-Pro-1 negative and M-540 positive were recorded as viable with high plasma membrane fluidity.

2.5.4. Intracellular ROS generation

Intracellular hydrogen peroxide (H_2O_2) production was measured to determine generation of ROS in viable sperm, using 5-(and-6) chloromethyl-20, 70-dichlorodihydro-fluorescein diacetate acetyl ester (CM- H_2DCFDA ; Life Technologies-Molecular Probes, USA) following the procedure described by Guthrie and Welch (2006). For each sperm sample, a 50 μL aliquot (30×10^6 spermatozoa/mL) was transferred to a culture tube containing 950 μL PBS, 1.25 μL H42 (0.05 mg/mL in PBS), 1 μL PI (0.5 mg/mL in PBS) and 1 μL H_2DCFDA (1 mM in DMSO). Then all samples were incubated at 37 °C in the dark for 30 min before flow cytometric analysis. The H_2DCF is oxidized by H_2O_2 to dichlorofluorescein (DCF), which fluoresces at 530/30 BP following excitation at 488 nm. A total of 10,000 sperm were recorded. Sperm being H42 positive, PI negative and DCF positive were recorded as viable with high intracellular H_2O_2 generation.

2.5.5. Early apoptosis-like changes

When early apoptotic-like changes occur, phosphatidylserine (PS) translocates from the inner to the outer leaflet of the sperm membrane (Peña et al., 2005). Annexin-V binds to sperm surface expressed PS. Thus, the detection of PS can be measured as the level of sperm with early apoptotic-like changes. Briefly, 45 μL of semen (20×10^6 sperm/mL) were mixed with 180 μL Annexin-V binding buffer and 5 μL H42 (0.05 mg/mL in PBS), 1 μL PI (0.5 mg/mL in PBS) and 3 μL Annexin V-FITC (Life Technologies-Molecular Probes, USA). After incubation in the dark at room temperature for 15 min, 200 μL Annexin-V binding buffer were added just before flow cytometric analysis. A total of 10,000 sperm were recorded. The sperm population displaying a PI negative and Annexin V-FITC positive

staining was recorded as of being viable sperm with early apoptotic-like changes.

2.5.6. Membrane lipid peroxidation

Sperm membrane lipid peroxidation was assessed using BODIPY 581/591 C11 (Life Technologies-Molecular Probes, USA) as described by Koppers et al. (2008) with slight modifications. Briefly, 1 mL of semen (20×10^6 sperm/mL) was incubated (at 37 °C for 30 min) with 2.5 µL BODIPY 581/591 C11 (diluted 1:1 w/v in ethanol). Then, the semen was washed once by centrifugation at 300 x g for 7 min. The supernatant was discarded and the pellet suspended in 1 mL PBS. Thereafter, 100 µL of the sperm sample were incubated (37 °C for 10 min) with 1.3 µL PI (0.5 mg/mL in PBS) and 2 µL H42 (0.05 mg/mL in PBS). Just before the flow cytometric analysis, 400 µL of PBS were added to each sample. A total of 10,000 sperm were recorded. The sperm population being H42 positive, PI negative and BODIPY positive was recorded as viable sperm with high sperm membrane lipid peroxidation.

2.6. Proteomic analysis

Once in the VTH-laboratory, the extended sperm pellets of P1 and P2 were centrifuged at 2,400 g for 3 min (Megafuge 1.0 R, Heraeus, Hanau, Germany) and the sperm pellets were re-extended in PBS to a concentration of $1,000 \times 10^6$ sperm/mL, aliquoted in 1 mL Eppendorf and stored at -80 °C until use. To avoid boar differences, a total of 12 sperm samples (3 boars; 4 ejaculates per boar) were mixed proportionally generating two single pools, one per P1 and another per P2.

2.6.1. Sperm protein extraction and quantification

The proteomic analyses were carried out in the Proteomics Unit of the University of Valencia, Valencia, Spain (member of the PRB2-ISCI ProteoRed Proteomics Platform). Sperm samples were thawed (rt) and then lysed for protein extraction in 200 μ L of U/T/C buffer [7 M Urea, 2 M thiourea and 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, (CHAPS)] during one hour with vigorous stirring. Proteins were precipitated with 2D-Clean up kit (GE Healthcare) and pellets were dissolved with buffer (7M Urea, 2M thiourea and 4% CHAPS, Tris 25 mM). One aliquot from each sperm protein sample was subjected to quantification by RCDC Lowry (Bio-Rad protein assay, Richmond, CA, USA) according to manufacturer instructions.

To reduce individual differences among ejaculate portions, sperm samples from each portion were mixed generating two single pools (P1 and P2). Fifty μ g of protein extract from each of the two pools were used for the electrophoresis analysis.

2.6.2. 2D-PAGE

After checking that the pH of all samples was between 8 and 8.5, the samples were labeled with the CyDye DIGE Fluor dyes following the labeling procedure recommended by GE Healthcare (GE Healthcare Life Sciences, Piscataway, NJ, USA). Briefly, 50 μ g protein from samples belonging to the P1 and P2 ejaculate portions were labeled with 400 pmol of Cy3 and Cy5, respectively, by incubating on ice and in the dark 50 μ g during 60 min. The labeling reaction was stopped by incubating the samples with 10 nmol of lysine for 10 min.

Each gel strip (24-cm Immobiline DryStrips linear pH 3 to 11 NL; GE Healthcare Life Science; Piscataway, NJ, USA.) was loaded by anodic cup-loading with a mix containing a total of 100 μ g proteins (50 μ g of proteins from P1 labeled with Cy3 and 50 μ g of proteins from P2 labeled with Cy5) diluted with DTT (65 mM) and 1% ampholyte solution. After

rehydration (8 M urea, 4% CHAPS, DeStreak reagent GE Healthcare (12 μ l/mL) and 1% (v/v) anpholites; pH: 3-11 NL) for 12 h, isoelectrofocusing (IEF; Ettan IPGphor II. GE Healthcare™) was performed as follows: 300 V 4 h, 1000 V 6 h, 8000 V 3 h, 8000V for 28000 Vh all at 20°C.

After completion of the IEF, strips were removed from the IEF instrument and equilibrated with equilibration buffer [50 mM Tris, 6 M urea, 30% (v/v) glycerol and 2% (v/v) SDS, pH 8.8 and 2% DTT (v/v)] for 15 min, and then with equilibration buffer containing iodoacetamide at a final concentration of 2.5% for 15 min with gentle shaking.

To run the second dimension, SDS-PAGE was performed on a 25 cm x 21 cm x 1 mm 12.5% polyacrylamide separating gel. Electrophoresis was performed at 2 w per gel during 1 hour followed by 15 w per gel during 5 h. (GE Healthcare™ Ettan DALTsix). Gel images were obtained using a Typhoon® 9400 Variable Mode Imager (GE Healthcare™) at the corresponding wavelength.

2.7. Statistical analysis

Statistics was performed using the IBM SPSS statistics 19.0 package (IBM Spain, Madrid). The Shapiro Wilk test was used to test normality. The Levene was used to check the homogeneity of the variances prior to run the ANOVA analysis. Mixed ANOVA (before freezing) and repeated measures ANOVA (after thawing) were performed using boar and ejaculate as random effects, to evaluate the influence of SP (own vs post-SRF) and sperm origin (first 10 mL of the SRF and rest of the SRF) on quality and sperm functionality. Results were showed as mean $\pm$ SEM and significance level was set to $P < 0.05$.

3. Results

3.1. Effect of SP on sperm freezability

Pre-freeze overnight incubation with SP from the post-SRF significantly altered ($P < 0.05$) the total and progressive motility of P1 and P2 spermatozoa compared to incubation with its own SP, while sperm viability was not influenced by incubation with SP from the post-SRF. However, significant differences ($P < 0.05$) in viability were found between P1 and P2 sperm, regardless of the SP used during pre-freezing incubation, with highest values obtained for spermatozoa belonging to P1 than that for those from P2 (See Table1).

After thawing, the SP source had no effect on any of the sperm parameters evaluated, regardless of whether they were evaluated at 30 or 150 min post-thawing. In contrast, the sperm source had a significant effect on sperm quality and the evaluated functional sperm parameters. There was no interaction between SP and sperm sources. Total and progressive motility and sperm viability (Figure 1) were higher ($P < 0.05$) and the percentages of viable sperm showing high plasma membrane fluidity and lipid peroxidation (Figure 2) were lower ($P < 0.05$) in P1 than P2, regardless of whether they were evaluated at 30 or 150 min post-thawing. In contrast, the percentage of viable sperm with early apoptosis-like changes (5.40 ± 0.40 vs 4.81 ± 0.25) and those generating ROS (48.55 ± 2.28 vs 47.43 ± 2.28) did not differ between P1 and P2.

3.2. Differences in sperm protein profiling between P1 and P2

A total of 1,930 resolved protein spots were generated in the gel image (Figure 3). Of these spots, 269 were differentially expressed in terms of fold-change (max fold change ≥ 2)

in normalized spot volume between P1 and P2, 145 spots being expressed more in P1 than in P2 (124 spots). Comparison analysis between protein spots of P1 and P2 showed that 36 protein spots were only present in P1 (green fluorescence) while 23 were exclusive of P2 (red fluorescence).

4. Discussion

The results showed that boar sperm from the SRF (either of the first 10 mL or the rest of SRF) incubated with SP from the post-SRF overnight impaired sperm quality, specifically motility parameters. These results were in agreement with those of a previous study demonstrating the detrimental effect on sperm motility when sperm from P1 were incubated with SP from entire ejaculate (Saravia et al., 2009). The negative effect of SP from the post-SRF on sperm motility has been attributed to its high concentration in bicarbonate and absence of some proteins with sperm-protective actions derived from epididymal fluid (Saravia et al. 2009). Moreover, Garcia et al. (2009) discouraged the utilization of SP from post-SRF as preservation agent for liquid storage of boar sperm because it impaired motility and viability, presumably related to its high concentration of PSP-I/PSP-II heterodimer. More recent studies (Barranco et al., 2105a and b), regarding the antioxidant capacity of SP, in terms of total antioxidant capacity (SP-TAC) and enzymatic antioxidant capacity, have demonstrated that SP from post-SRF have lower SP-TAC and Paraoxonase type 1 activity than the SP from SRF, including that of first 10 mL of SRF (P1). In addition, these studies have revealed that this reduced SP-antioxidant capacity of post-SRF affected negatively boar motility and sperm viability in liquid semen samples storage up to 72h. Taken together, the above studies seems to indicate that the SP of the post-SRF has detrimental effects on boar sperm during liquid

storage, mainly due to its low antioxidant content and its protein composition. A recent proteome study of boar SP identified 34 proteins showing quantitative differences between SRF and post-SRF (Perez-Patiño et al., 2016). These findings should be considered when deciding whether to collect the entire ejaculate or just the SRF for either production of liquid AI-semen doses or for sperm cryopreservation.

Incubation of sperm with SP overnight before freezing evidenced that sperm from P1 showed higher percentages of viable than that of P2 after incubation, regardless of the PS used for incubation. In this regard, Saravia et al. (2009) demonstrated that sperm from P1 were characterized by having a highly stable plasma membrane, which would be related to the dominating epididymal fluid they were surrounded by (Siquiera et al., 2011). In agreement, Dacheux et al., (2012) suggested that major proteins interacting with sperm during its passage across the epididymis are relevant to sperm for better sustaining preservation.

Post-thaw analysis of sperm quality and functionality showed that P1 sperm displayed a better sperm cryosurvival than those of P2, regardless of the SP source used for overnight pre-freezing incubation. Specifically, sperm from P1 showed better post-thaw motility and viability and lower plasma membrane fluidity and lipid peroxidation than those of P2. These findings are surprising since they contradict the widespread and accepted concept that the SP from post-SRF is detrimental to boar sperm freezability (Saravia et al., 2009; Alkmin et al., 2014). These findings would indicate that, under our experimental conditions, the differences among ejaculate fractions/portions on boar sperm freezability would be more intrinsically linked to sperm structure than SP composition. Alternatively, the results might indicate that the first adsorption of the surrounding SP could have a stronger effect than expected. To the

best of our knowledge this is the first study showing the relevance of sperm origin on cryopreservation success. Previous studies (Siqueira et al. 2011; Alkmin et al. 2014) did not found differences in the ability of sperm from P1 and P2 portions to tolerate freezing-thawing procedure. A possibility to explanation of this disagreement would be related with the manipulation of the sperm made in our study that included the separation of them from SP by centrifugation immediately after ejaculation and subsequent re-extension of them with SP from various sources. In any case, our study reveals that beyond the influence of SP, there could be structural differences between the spermatozoa of the different fractions that would determine their response to cryopreservation. Kumaresan et al. (2011) suggested differences between P1 and P2 in the ability of sperm to undergo protein tyrosine phosphorylation, which influenced sperm cryoresistance.

In the light of the aforementioned, it is reasonably to suggest that the differences in the sperm composition of P1 and P2 are relevant for freezability success. Accordingly, the protein profiles of boar spermatozoa from P1 and P2 were evaluated using a 2D-PAGE procedure. The results were, to the best of our knowledge, the first reporting differences in protein composition between boar spermatozoa of different ejaculate fractions/portions, and they highlighted a putative relevance of such differences on the ability of sperm to successful sustaining cryopreservation. Previous studies demonstrated quantitative differences in some specific sperm proteins between boar semen ejaculates showing good and bad sperm freezability (Vilagran et al. 2013, 2014; Guimaraes et al. 2017). Furthermore, Chen et al. (2014) demonstrated changes in sperm proteome during the cryopreservation process, suggesting that sperm proteins may play an important role for cryosurvival. Probably, the SP surrounding sperm is not a passive subject in the differences observed in the sperm proteome

profile between P1 and P2. In this regard, it is well known that there is protein exchange between sperm and SP during ejaculation which apparently occurs instantaneously and remains effective thereafter (Leahy and Gadella, 2011). It is also important to remember that the spermatozoa of the first 10 mL (P1) interact mainly with epididymal fluids and those of P2 interact more with secretions from the prostate and seminal vesicles (Rodriguez-Martinez et al. 2011). Our results should be considered as preliminary and, therefore, further studies including deep knowledge of sperm proteome are needed to evidence the magnitude of differences among ejaculate fractions/portions in sperm protein composition and to then analyze the relevance of different proteins for sperm freezability. Moreover, it should be imperative to confirm if differences in protein profiling between sperm from P1 and P2 are inherent to the spermatozoa or whether they are linked to the putative structural changes experienced by sperm during ejaculation in relation to the interaction between sperm and epididymal fluids and/or SP.

In conclusion, the results of the present study demonstrated a motility impairment in overnight pre-freezing incubated sperm with SP from post-SRF, although this negative effect, surprisingly, does not impair the subsequent sperm freezability. The results also showed that differences between ejaculate fractions/portions on boar sperm freezability would be more intrinsically linked to sperm characteristics than SP composition. This statement seems supported by the evident differences on sperm protein profile between ejaculate fractions/portions. Further studies involving full proteome characterization of boar spermatozoa, including protein identification and quantification, are essential to reveal the identity of sperm proteins different between ejaculate fractions/portions, as well as to demonstrate their potential implication in sperm freezability.

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Table 1. Sperm motility (total and progressive) and sperm viability pre-freezing in fresh sperm samples from the first 10 mL of sperm rich ejaculate fraction (P1) and the rest of sperm rich ejaculate fraction (P2) re-extended in their own SP or with SP from the post-ejaculate fraction (Post-SRF) and incubated at 17°C overnight.

Treatment	Sperm quality (%)		
	Total motility	Progressive motility	Viability
Sperm from P1 + SP from P1	78.60 ± 4.08 ^a	40.20 ± 3.12 ^a	88.29 ± 0.98 ^a
Sperm from P1 + SP from post-SRF	71.40 ± 2.80 ^b	36.80 ± 3.73 ^b	89.64 ± 0.56 ^a
Sperm from P2 + SP from P2	78.10 ± 3.28 ^a	41.20 ± 3.59 ^a	87.59 ± 1.03 ^b
Sperm from P2 + SP from post-SRF	74.00 ± 4.23 ^b	35.80 ± 3.47 ^b	86.78 ± 0.86 ^b

Data are expressed as means ± SEM. ^{a,b} indicate difference among treatments (P < 0.05)

Figure Legends

Figure 1. Sperm motility (total and progressive) and sperm viability in frozen-thawed sperm samples from the first 10 mL of the sperm rich ejaculate fraction (P1, white bars) and the rest of the sperm rich ejaculate fraction (P2, grey bars). Data are expressed as means $\pm$ SEM. a, b and x, y indicate differences $P < 0.05$ and $P < 0.01$, respectively.

Figure 2. Percentage (means $\pm$ SEM) of viable sperm with high membrane fluidity and lipid peroxidation in frozen-thawed sperm samples from the first 10 mL of the sperm rich ejaculate fraction (P1, white bar) and the rest of sperm rich ejaculate fraction (P2, grey bar). a, b indicate difference ($P < 0.05$).

Figure 3. A: Gel images showing protein expression of sperm from the first 10 ml of sperm rich ejaculate fraction (P1, labelled with Cy3) and the rest of sperm rich ejaculate fraction (P2, labelled with Cy5). B: Overlap image of the two gel images showing shared protein spots (orange or yellow) and protein spots only present in the sperm from the first 10 ml of sperm rich fraction (P1, green spots, marked by green squares) and those only present in the sperm from the rest of sperm rich fraction (P2, red spots, marked by red triangles). All the gels were run with a pH range 3-11 from left to right in the horizontal dimension and molecular weight range from 10-250 kDa from bottom to top in the vertical dimension. Gel images were obtained using a Typhoon® 9400 Variable Mode Imager (GE Healthcare™) at the corresponding wavelength.

Percentage

100

80

60

40

20

0

Total motility

Progressive motility

Viability

a

b

x

y

x

y

**59.25
±1.34**

**54.75
±1.60**

**44.53
±1.24**

**40.16
±1.25**

**65.95
±0.65**

**57.25
±0.99**

Percentage

12

10

8

6

4

2

0

Plasma membrane fluidity

b

4.20
±0.40

a

6.20
±0.84

Membrane lipid peroxidation

y

4.91
±0.50

x

9.29
±0.86

Cy3

Cy5

A

B