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Boar spermatozoa from the sperm-peak portion of the ejaculate show similar sperm quality after simplified freezing in MiniFlatpacks as those in the rest of the sperm-rich fraction, bathing in seminal plasma of similar proteomic profile

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Short title: Freezability of boar ejaculate portions.

Key words: cryopreservation, ejaculate portions, sperm kinematics, membrane intactness, pig.

Abstract

Boar sperm viability post-thaw differs depending on the ejaculate fraction used, with spermatozoa present in the first 10mL of the sperm-rich fraction, SRF (portion 1, P1, sperm-peak portion), showing the best cryosurvival *in vitro* when compared with spermatozoa from the rest of the ejaculate (second portion of the SRF plus the post-spermiatic fraction), even when using simplified freezing routines. Such abilities apparently relate to the specific composition of seminal plasma in the P1 (glycoproteins, pH, and bicarbonate concentrations). However, spermatozoa from P1 have not been compared to the rest of spermatozoa in the SRF (SRF-P1, usually 30 to 40 mL of the SRF), which is routinely used for freezing. Such comparison of P1 *vs* SRF-P1 was hereby done, in terms of sperm kinematics (QualiSperm™ system), membrane integrity (SYBR-14/PI), acrosome integrity (FITC PNA/PI), and sperm membrane stability (Annexin-V), explored using flow cytometry. As well, total protein concentration and the proteomics of the seminal plasma (SP) of either portion of the SRF were studied using two-dimensional electrophoresis (2DE), mass fingerprinting (MALDI-TOF) and collision-induced dissociation tandem mass spectrometry (CID- MS/MS) of selected peptides. The SRF portions were weekly collected from four mature boars (4-5 replicates per boar, sperm concentration: P1- 1.86 ± 0.20 , SRF-P1- $1.25 \pm 0.14 \times 10^9$ spz/mL) and processed using a quick freezing method in MiniFlatPacks (MFP). Sperm motility post-thaw reached 50%, without differences between portions of the SRF, but with clear inter-boar variation. Neither did plasma membrane or acrosome integrity differ (ns) between fractions. These results indicate that there are no differences in cryosurvival after quick freezing of boar spermatozoa derived from either of the two portions of the SRF. While P1 and SRF-P1 clearly differed in relative total protein contents, as expected, they displayed very similar protein profiles as assessed by 2DE and mass spectrometry (tryptic peptide mass fingerprint analysis and CID-MS/MS), indicating a similar emission of epididymal protein content.

1. Introduction

The boar ejaculate consists of several consecutive jets expelled in a fractionated way, with up to three fractions easily recognizable by their appearance and density. The first fraction is watery being called the pre-sperm fraction (PSF) owing to its absence of spermatozoa. The second fraction is called sperm-rich fraction (SRF) where the bulk of spermatozoa is verted, particularly at the beginning (the so-called sperm-peak portion, or P1 a 10-15 mL portion whose contents of epididymal caudal fluid is high; Rodriguez-Martinez et al, 2009). The rest of the SRF continues having high numbers of spermatozoa mixed with a richer protein secretion from the vesicular glands (Einarsson, 1971). The post-sperm-rich fraction (PSRF) has decreasing sperm numbers and increasing secretions of vesicular, prostate and, by the end of ejaculation, bulbourethral glands (Mann & Lutwak-Mann, 1981).

The seminal plasma (SP) of boars consists of many components that influence, inhibiting or stimulating, a multitude of sperm functions, the female genital tract during sperm transport (Rozeboom et al, 2000; O'Leary et al, 2004; 2006) and events preceding fertilization (Rodríguez-Martínez et al, 2005; Maxwell et al, 2007). Thus it is not surprising that certain amounts of SP present in slightly cooled or frozen semen can improve sperm motility (Rodriguez-Martinez, 1991), maintain acrosome integrity (Maxwell et al, 1997), delay capacitation-like changes (Pursel et al, 1973), and increase the resistance to cold shock (Pursel et al, 1973; Watson, 2000) or oxidative stress (Roca et al, 2004; 2005). Maintenance of these sperm attributes obviously lead to higher sperm longevity, cryosurvival and, subsequently, fertility (Zhu et al, 2000; Hernández et al, 2007; Okazaki et al, 2009; Garcia et al, 2010). However, spermatozoa are not exposed to a mixture of SP fractions *in vivo*, as it occurs when an ejaculate is collected (Rodriguez-Martinez et al, 2005), and SP has been considered detrimental for sperm viability if the spermatozoa are singly stored in SP (Kawano et al, 2004). Therefore, removal (by extension and/or centrifugation) of SP has been customary during preservation. Yet, it is well known that about 70% of variability in pig sperm cryosurvival can be attributed to male effects (Roca et al, 2006) where SP protein characteristics differ among boars (Flowers and Turner, 2001; Hernandez et al, 2007).

Cryopreservation of boar semen has advanced over the past decade, with inclusion of various additives in the semen extender (Peña et al, 2003a, 2004; Roca et al, 2004, 2005), novel packaging systems (Eriksson & Rodriguez-Martinez, 2000; Saravia et al, 2009), and simplified freezing protocols (Saravia

et al, 2010). The latter was possible by findings in this laboratory, which demonstrated that the sperm-peak portion of the ejaculate (the first 10 mL of the SRF, P1) can equally sustain conventional or simplified cryopreservation (Saravia et al, 2005; 2010) and -when this is done- more than 50% of the time allotted for conventional freezing is saved, avoiding extension or centrifugation. The spermatozoa present in P1 were superior in sustaining viability *in vitro* when compared with the spermatozoa from the rest of the ejaculate (portion 2, second portion of the SRF plus the PSRF), showing virtually no variation between boars (Saravia et al, 2010). Moreover, use of MiniFlatPacks™ (MFP) to pack and freeze the P1 in single AI doses for deep intrauterine insemination (Wongtawan et al, 2006) increased our capability to make more rational use of the ejaculate (Saravia et al, 2010).

Differences in the type and amounts of ions, proteins, bicarbonate and pH, reminders of the milieu of the cauda epididymides, have been considered as crucial for this better cryosurvival of P1 spermatozoa (Saravia et al, 2009; Rodriguez-Martinez et al, 2009). Cross-exposure of spermatozoa from either portion (P1 vs P2) to their native SP could clearly influence their cryosurvival, thus reinforcing the above concept: particular portions of the SP modulate sperm structure, intactness and, ultimately, function (Saravia et al, 2009). Specifically, differences in SP proteomics have been described (Rodríguez-Martínez et al, 2005; Saravia et al, 2009) and different SP protein profiles have been found between boars of different *in vivo* fertility (Flowers and Turner, 2001). Whether there are significant differences in proteomics between portions of the SRF, regarding SP-proteins related to sperm viability or fertility remains to be studied.

Under current conditions of boar semen preservation, primarily aiming the use of most spermatozoa without incurring in difficult handling, the SRF is the fraction collected and used, and SP is routinely extended prior to cooling to +15°C, and then removed usually a couple of hours later by centrifugation. Although examined against the rest of the ejaculate without removal of the accompanying native SP (Saravia et al, 2010), spermatozoa from P1 have not yet been compared to the rest of the spermatozoa in the SRF (SRF minus P1, usually 30 to 40 mL of the SRF) for eventual differences in cryosurvival and SP-protein composition when using simplified freezing.

The present experiment sought for eventual differences in cryosurvival between spermatozoa present in either portion, in terms of sperm kinematics (QualiSperm™ system), membrane integrity (SYBR-14/PI),

acrosome integrity (FITC PNA/PI), and sperm membrane stability (Annexin-V), explored using flow cytometry (FC). As well, the proteomics of the SP of either portion of the SRF was explored using two-dimensional electrophoresis (2DE), mass fingerprinting (MALDI-TOF) and collision-induced dissociation tandem mass spectrometry (CID- MS/MS) of selected peptides.

2. Material and methods

2.1. Animals

Four mature boars (1.5-4 years old) were used (one Swedish Landrace, two Swedish Yorkshire, and one Norwegian Landrace), selected for clinical normality, acceptable semen quality and fertility after AI with liquid semen. No boar was pre-selected for semen freezability. Boars were kept on straw beds in individual pens at the Division of Reproduction, SLU, Uppsala, Sweden; fed according to Swedish standards (Simonsson, 1994) and provided with water *ad libitum*. The experimental protocol had previously been approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

2.2. Semen collection and primary processing

Ejaculates were collected once weekly, during 4-5 consecutive weeks from each boar. Collection was done manually (gloved-hand technique) to enable partition of the SRF. The first 10mL of the SRF (portion 1, P1) and the rest of the SRF (SRF-P1, restricted to 30-40mL) were collected into pre-warmed 12 or 50mL graduated plastic tubes, respectively. Only ejaculates with $\geq 70\%$ motile spermatozoa and $\geq 80\%$ morphologically normal spermatozoa were used. Sperm concentration was manually assessed with a Bürker chamber (Bane, 1952). The total numbers of spermatozoa in P1 and SRF-P1 were calculated by multiplying sperm concentration/mL by volume of collected aliquots of the SRF and expressed as 10^9 spermatozoa.

2.3. Seminal plasma collection and storage

SP-samples obtained from the same four boars were used for protein content and peptide profile determinations. The collected SRF portions (P1 and SRF-P1) were centrifuged five times ($3,000 \times g / 20 \text{ min}$) with the SP supernatant moved to a clean tube before each consequent centrifugation. After the last centrifugation, the harvested SP (from P1 and SRF-P1) was separately filtered through disposable filters of $0.2\mu\text{m}$ diameter (Filtropur S; Sarstedt AG & Co, Nümbrecht, Germany), put into 10mL tubes, separately for each boar and portion and stored at -20°C until analysis.

2.4. Semen freezing

Both ejaculate portions were processed separately for cryopreservation following existing protocols for MiniFlatPacks (MFPs), the so-called “simplified freezing method” (Saravia et al, 2010). In brief, both spermatozoa from P1 or SRF-P1 were held in their own SP in the dark at room temperature ($+20\text{-}22^\circ \text{C}$) for at least 30 min. After that period, the semen was mixed with a lactose-egg yolk (LEY) extender at a ratio of one to two parts semen to one part extender, and cooled to 5°C within 1.5h. The LEY-extender semen was then slowly mixed with LEYGO extender (at a ratio of two parts of semen to one part of extender - to yield a final sperm concentration of about 1.0×10^9 spermatozoa/mL). The LEYGO was composed of LEY, Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA; [equivalent to Orvus Es Paste, Graham et al, 1971]) and glycerol (3% final proportion). Spermatozoa were then transferred to a cool cabinet at 5°C (IMV, L’Aigle, France) and packaged in MFPs each holding $\sim 500 \times 10^6$ spermatozoa. The MFPs were heat-sealed and cooled/frozen with a programmable freezer (Mini Digitcool 1400; IMV, L’Aigle, France) at rates of $3^\circ \text{C}/\text{min}$ from 5 to -5°C , 1 min was allowed for crystallization, and thereafter cooled at $50^\circ \text{C}/\text{min}$ from -5°C to -140°C . The MFP were then plunged into liquid N_2 (-196°C) for storage before final thawing in circulating water at 35°C for 20s.

2.5. Sperm analyses

2.5.1 Sperm motility

Sperm motility was evaluated on fresh and thawed/incubated ($38^\circ\text{C}/30\text{min}$) semen. Three motility parameters (total motility [%], progressive motility [%] and sperm velocity [$\mu\text{m}/\text{s}$]) were determined using the QualiSpermTM system, version 1.3 (Biophos, Pfäffikon, Switzerland). After thawing, the semen suspension was extended with BTS+LEY (95mL BTS+5mL LEY) at a 1:5 to 1:10 ratio, to give a

sperm concentration of about $40\text{-}50 \times 10^6$ spermatozoa/mL and warmed at 38°C for 30 min. Following gentle mixing, five μL of each sample were placed in a pre-warmed (38°C) $10\mu\text{m}$ -deep Markler chamber (Sefi Medical Instruments, Haifa, Israel). Sperm movement was assessed using a Nikon E200 microscope (Nikon, Tokyo, Japan) equipped with phase contrast optics and a thermal plate, at 100x magnification. The spermatozoa were recorded in one field at a rate of 50 frames/s (200 frames in total) using a MVD640- 48-U2-10 Photon Focus camera (Photon Focus, AG, Lachen, Switzerland). The data recorded were the proportions of motile, progressive motile and immotile spermatozoa, and their mean speed. Additionally, classes of sperm velocity ($\mu\text{m/s}$) in post-thawing samples were recorded; spermatozoa being distributed (%) in 11 velocity classes: 0= $0\mu\text{m/s}$ (Immotile); A= $0\text{-}10\mu\text{m/s}$ (Non-progressive); B= $10\text{-}35\mu\text{m/s}$; C= $35\text{-}40\mu\text{m/s}$; D= $40\text{-}45\mu\text{m/s}$; E= $45\text{-}50\mu\text{m/s}$; F= $50\text{-}55\mu\text{m/s}$; G= $55\text{-}60\mu\text{m/s}$; H= $60\text{-}70\mu\text{m/s}$; I= $70\text{-}75\mu\text{m/s}$; J= $>75\mu\text{m/s}$.

2.5.2 Sperm plasma membrane integrity and stability, and acrosome integrity

These measurements were performed using an LSR FC (Becton Dickinson, San José, CA, USA) following established protocols (as described in Saravia et al, 2007). *Sperm plasma membrane integrity* (PMI) was assessed using the LIVE/DEAD[®] Sperm Viability Kit L-7011 (Molecular Probes Inc., Eugene, OR, USA). Frozen semen samples were thawed and suspended in prewarmed BTS[®] ($2\mu\text{L}$ semen to $500\mu\text{L}$ BTS). This suspension was centrifuged (10min, 25°C , $300 \times g$) and the pellet resuspended in 1mL of BTS. The sperm suspension were loaded with $1\mu\text{L}$ SYBR-14 (1:50) and $5\mu\text{L}$ propidium iodide (PI) fluorophores and incubated at 37°C for at least 10 min before FC-analysis. The data from at least 10,000 gated events per sample were collected in list mode, and spermatozoa classified as live (SYBR+/PI-), dying (SYBR+/PI+), or dead (SYBR-/PI+) (as described in Saravia et al, 2005). *Acrosome intactness* was assessed with the acrosome-specific FITC-labelled PNA in combination with propidium iodide (PI) and H33342 (Molecular Probes Inc., Eugene, OR, USA). Briefly, spermatozoa were stained with PI ($2.5\mu\text{L}$), Ho3342 ($1\mu\text{L}$) and FITC PNA ($5\mu\text{L}$), incubated for 10 minutes at 37°C , and analyzed as described in Spjuth et al. (2007). Viable spermatozoa (PI-negative, PI-) with an intact outer acrosome membrane were PNA-FITC-positive (PNA-FITC+), while PNA-FITC-negative (PNA-FITC-) spermatozoa were considered acrosome-exocytosed. *Sperm membrane architecture and early destabilization* were evaluated by probing the spermatozoa with Annexin-V/PI as described by Saravia et al. (2009) using an Annexin-V-FITC apoptosis detection kit I and II (Pharmingen, San Diego, CA, USA) (Peña et al, 2003a). Frozen semen samples were thawed and an aliquot of $10\mu\text{L}$

was extended with 90µL of binding buffer. To this suspension, 90µL of binding buffer, 3.5µL Annexin, 10µL PI and 2µL Ho342 (500µg/mL) were added before incubation in darkness at room temperature for 15 min. Extra 400µL of binding buffer were added before FC-analysis. For the gated cells, the percentages of viable spermatozoa with a stable plasmalemma [Annexin-V (AN)-negative/PI-negative (PI-)], spermatozoa with an unstable yet intact plasma membrane (AN+/PI-), and membrane-damaged cells (AN-/PI+), as well as double positive (AN+/PI+) cells were evaluated based on quadrants determined from single-stained and unstained control samples.

2.6. Seminal plasma proteomics

2.6.1 Total protein concentration

SP-samples were thawed at room temperature (20-22°C) and the total protein concentration was quantified by the Bradford (1976) spectrophotometric method, using bovine serum albumin (BSA, 1mg/mL) to plot a standard curve, and readings at 595nm absorbance. A linear regression equation was used to determine the total protein concentration in the SP-samples.

2.6.1. Gel electrophoresis of seminal plasma

The SP-protein profile of each sample was determined using two-dimensional electrophoresis (2DE). For running the first dimension (isoelectric focusing), seven-cm long IPG strips (Bio-Rad), pH range 3-10, were rehydrated in 150 µL of protein solution (containing a total of ca. 200 mg of protein), for 4 h at room temperature. Isoelectric focusing (IEF) was carried out with a Protean IEF Cell (Bio-Rad) using the following conditions: 1h at 500V, 2.5h at 1,000V and 2.5h at 5,000V. After focusing, strips were equilibrated in 50mM Tris-HCl, 6M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 1% (w/v) DTT and 2.5% iodoacetamide for 15 min. The IPG strips were then laid on 15% SDS-PAGE gel slabs with 0.5% agarose in the cathodic buffer (192 mM glycine, 0.1% SDS and Tris-HCl to pH 8.3). The electrophoretic run was performed until the dye front reached the gel bottom. Gels were incubated in a colloidal Coomassie Blue solution (EasyStain, Invitrogen), and destained in deionized water.

2.6.2 *In-gel enzymatic digestion and mass fingerprinting*

Protein bands of interest were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to automated reduction with DTT and alkylation with iodoacetamide, and in-gel digestion with sequencing-grade bovine pancreas trypsin (Roche) using a ProGest digester (Genomic Solutions) following the manufacturer's instructions. About 0.65 mL of the tryptic peptide mixtures (total volume of ~ 20 µL) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α -cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes. A tryptic peptide mixture of *Cratylia floribunda* seed lectin (SwissProt accession code P81517) prepared and previously characterized in our laboratory was used as mass calibration standard (mass range, 450-3300 Da).

2.6.3. *Collision-induced dissociation tandem mass spectrometry (CID- MS/MS)*

For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization (ESI) mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems) equipped with a nanospray source (Protana, Denmark). Doubly- or triply-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode and the mono-isotopic ions were fragmented using the Enhanced Product Ion tool with Q_0 trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for MS/MS experiments were as follows: Q1- unit resolution; Q1-to-Q2 collision energy - 30-40 eV; Q3 entry barrier - 8 V; LIT (linear ion trap) Q3 fill time - 250 ms; and Q3 scan rate - 1000 amu/s. CID spectra were interpreted manually or using a licensed version of the MASCOT program (<http://www.matrixscience.com>) against the SwissProt/TrEMBL database (UniProtKB/Swiss-Prot Database; <http://us.expasy.org/sprot/>).

2.7. Statistical analysis

Differences in mean values for sperm parameters (using arcsine transformation where data did not show normal distribution) were examined by analysis of variance (ANOVA) using the General Linear Models procedure of the SPSS statistical package, version 17.0 (SPSS Inc., Chicago, USA). The statistical model included the fixed effects of male, stage (fresh or post-thawing) and portion within SRF-fraction

and their interaction. When overall significance was found, comparisons were done using SNK or *t* student test. The results are presented as mean±SEM and the level of significance was set at $P < 0.05$. Also, we carried out a correlation analysis (Spearman) between pre-freezing variables (excluding those derived from the subpopulation study) and post-thaw variables.

3. Results

The P1 had a mean sperm concentration of $1.86 \pm 0.20 \times 10^9$ spermatozoa/mL (\pm SEM, $n=19$) which significantly differed from SRF-P1 ($1.25 \pm 0.14 \times 10^9$ spermatozoa/mL, $P < 0.05$), both SRF-portions varying among boars ($P < 0.01$). The total volume and concentration per male and SRF-portion are depicted in Table 1.

Motility parameters in both SRF-portions (P1 and SRF-P1) for fresh and frozen-thawed samples are shown in Table 2. There were no differences between portions for any parameter analyzed in fresh semen, but there was variation among boars for total motility ($82.8 \pm 3.7\%$ up to $90.6 \pm 4.7\%$, $P < 0.05$).

Post-thaw, total sperm motility, progressive motility and velocity reached means (\pm SEM) of $47.82 \pm 2.69\%$, $31.86 \pm 2.40\%$ and $32.81 \pm 2.13 \mu/s$ for P1 and $47.23 \pm 3.68\%$, $32.19 \pm 3.26\%$ and $32.74 \pm 2.74 \mu/s$ for SRF-P1, respectively. There were no significant differences between SRF-portions (ns), but with clear inter-boar variation ($P < 0.01$). Boar D showed the best results with regards to total motility post-thaw (P1, $54.75 \pm 3.63\%$; SRF-P1, $59.78 \pm 3.63\%$) while boar B showed the worst results (P1, 32.2 ± 3.6 ; SRF-P1, 25.6 ± 1.8).

The speed sperm classification did not show differences between SRF portions (Fig. 1; $P > 0.05$), but differed among animals, particularly between boars B and D for all classes investigated ($P < 0.05$). Boar B displayed the lower percentage of spermatozoa ($6.5 \pm 1.3\%$) in the rapid progressive class (K), whereas boar D showed the higher percentage of spermatozoa ($23.09 \pm 1.2\%$) in the rapid progressive class, thus obviously correlating with total motility ($r^2=0.91$) and progressive motility ($r^2=0.94$). On the other hand, there was no significant correlation ($P > 0.01$) between fresh and post-thawing parameters analyzed.

The proportions of spermatozoa with an intact plasma membrane post-thaw, as assessed by SYBR-14/PI, are shown in Table 3. There were no significant differences ($P>0.05$) in percentages of spermatozoa with intact plasma membrane between P1 and SRF-P1 (54.82 ± 2.92 vs 55.49 ± 2.15). Likewise, there were no significant differences ($P>0.05$) in percentages of viable spermatozoa with plasma membrane stability post-thaw between P1 and SRF-P1 (50.80 ± 1.91 vs $51.67\pm 1.63\%$). The data of the assessment of early sperm plasma membrane destabilization after thawing, using Annexin-V and PI are depicted in Table 4. Acrosome integrity post-thawing is shown in Table 5. The rate of live-acrosome intact spermatozoa, was similar between SRF-portions (58.52 ± 2.38 vs 58.23 ± 2.14 , respectively for P1 and SRF-P1, $P>0.05$).

There were differences among boars for all measurements described above ($P<0.05$). Surprisingly, boar B, depicting the worst sperm kinematic values post-thaw, showed the best results with relation to sperm plasma membrane integrity ($63.49\pm 2.74\%$), stability ($56.39\pm 2.01\%$) and acrosome integrity ($63.35\pm 2.75\%$).

The SRF-portions differed significantly in their relative total protein contents, being significantly higher in SRF-P1 (39.8 ± 5.12 g/L; $P<0.05$) than in P1 (21.71 ± 4.46 g/L), as expected. However, 2DE analysis (Fig. 2) clearly revealed that the two portions of the SRF (left panel: P1; right panel: SRF-P1) displayed almost indistinguishable protein compositions. This point was confirmed by tryptic peptide mass fingerprinting (PMF) of spots displaying the same isoelectric point and apparent molecular mass in the P1 and SRF-P1 2DE gels. Protein identifications by PMF were further validated by CID-MS/MS analysis. The major proteins shared between these fractions are labeled with same numbers in the panels of Fig. 2. In particular, MS/MS analysis of the following tryptic peptide ions identified spot 1 as the porcine homolog of the fatty acid binding protein lipocalin-9 from *Equus caballus* [NCBI Reference Sequence: XP_001917526.1] (584.6^{2+} , GTPXANGDXAXK; 611.6^{3+} , GAVDGQFSNAAXAQTDXR; 559.9^{2+} , NAWXQXFAR; X= Leu or Ile), and spots 2 and 3 as boar epididymal secretory protein-1 [O97763] (784.4^{2+} , DQTYSYLNKLPVK; 508.5^{2+} , SGINCPIQR). Spots 5-10 corresponded to boar spermadhesin PSP-I [P35495] isoforms (718.3^{2+} , LTDDYGTIFTYK; 524.8^{2+} , LDYHACGGR; 567.6^{3+} , DSGHPASPYEIIIFLR; 604.8^{2+} , FCEGLSILNR). In addition, porcine prostaglandin D synthase [Q765P8] (675.3^{2+} , GFTEDGIVFLPR; 638.6^{3+} , NYALLHTESGSPGPAFR) was found in a 1D SDS-PAGE separations of P1 and SRF-P1 proteins.

4. Discussion

The development of a commercially acceptable design to cryopreserve boar semen has been pursued. In particular, a method that produces an unique AI dose of small volume albeit of high sperm concentration and high cryosurvival, yet consuming less time than at present, and thus allowing for a co-processing under routine conditions of commercial production of liquid semen would be in the best interest of the swine industry, provided these small volume doses could be inseminated intra-uterus yielding acceptable farrowing rates and litter sizes..

Use of a simplified-short freezing protocol (Saravia et al, 2010) in combination with a well-defined portion of the boar ejaculate, e.g. the sperm peak-portion of the SRF (P1), appeared suitable for the more extensive use of frozen-thawed boar semen, since this protocol reduced the time involved in the handling of the semen (8-9 h are usually required to process an ejaculate using the current technique), diminished the inter-boar variability in the cryopreservation (probably as a concerted action of by the SP-composition of the P1 and the packing in cryobiologically advantageous containers such as the MFP; Saravia et al, 2009), and allow to use the rest of the ejaculate to produce routine liquid semen doses for conventional AI (Saravia et al, 2010).

In the present study, two portions of the SRF (P1 vs SRF-P1) were compared using the same method for simplified cryopreservation as Saravia et al. (2010), intending to disclose whether either one could be indistinctly used. The sperm concentration in P1 represented the sperm-peak with almost 30% of the total number of spermatozoa that constituted the SRF, thus confirming previous data in other boars (Saravia et al, 2010). Additionally, the potential number of doses (with 1×10^9 spermatozoa/mL) for each fraction was 19 from P1 and 46 from SRF-P1, thus reinforcing the idea that the use of P1-portion processed by this newly simplified freezing protocol, produces a sufficient number of doses that would allow the swine industry to build genetic banking without compromising the commercial routine of the boar stud (Saravia et al, 2010).

Sperm kinematics (% total motility, % progressive motility and sperm velocity) displayed by the spermatozoa present in either portion of the SRF differed ($P<0.05$) among boars, ranging 30 to 60% of total motile spermatozoa, but without differences when SRF-portions were compared. Male B showed the poorest motility post-thaw for both portions studied. Therefore, an aliquot of each SRF-portion of this male was thawed and incubated in the presence of 5mM caffeine (Sigma Chemical Co., USA). The addition of caffeine significantly ($P<0.05$) increased total motility (P1, 32.2 ± 3.6 vs $67.9\pm 0.6\%$; SRF-P1, 25.6 ± 1.8 vs $61.0\pm 4.2\%$), as well as progressive motility (P1, 19.1 ± 2.2 vs $55.5\pm 0.7\%$; SRF-P1, 15.9 ± 1.7 vs $45.9\pm 3.3\%$) and mean sperm velocity (P1, 27.6 ± 2.2 vs 61.2 ± 2.9 $\mu\text{m/s}$; SRF-P1, 25.5 ± 1.9 vs 43.4 ± 3.1 $\mu\text{m/s}$) in either SRF-portion (n.s).

Differences in sperm quality post-thaw between boars were sometimes found (Peña et al, 2006) or not (Saravia et al, 2007). Inter-animal differences in their ability to sustain sperm cryopreservation had been associated to the SP-composition (Flowers, 2001; Novak et al, 2010), genetic factors (Thurston et al, 2002), or by the existence of distinct spermatozoa subpopulations in the ejaculate, in terms of their morphological characteristics (Thurston et al, 2001) or movement patterns (Cremades et al, 2005).

In this context, we ranked the different speed classes of the frozen-thawed boar spermatozoa present in those distinct SRF-portions to define sperm populations by cryosurvival. In general terms, there was no difference between portions within the SRF-fraction, but again, there was a clear difference among boars. Male B, which showed the poorest post-thaw motility also showed the fewest spermatozoa depicting rapid progressive motility ($>75\mu\text{m/s}$). There is obviously a need for testing a larger number of boars, correlating the different speed sperm populations on its subsequent *in vivo* or *in vitro* fertility to determine whether the impaired fertility of cryopreserved semen is due to insufficient numbers of spermatozoa in a specific subpopulation of the ejaculate (Rodríguez-Martínez, 2007).

Previous results confirmed the existence of essential differences between the P1 and the rest of the ejaculate (Rodríguez-Martínez et al, 2009; Saravia et al, 2009) related to types and amounts of ions, proteins, bicarbonate and pH indicating that the P1 spermatozoa were still bathing in a substantial amount of fluid from the cauda epididymides, in which they were emitted at ejaculation, and that this would explain the better performance of the spermatozoa contained in this SRF-portion. The findings of the current study, have clearly shown that despite the SRF-P1 having a significantly larger relative

contents of total protein than the P1, these portions of the SRF displayed very similar protein profiles as assessed by 2DE and mass spectrometry (tryptic peptide mass fingerprint analysis and CID-MS/MS) (Fig.2). Of particular interest was the similar display of epididymal-contained proteins (such as the Lipocalin-type Prostaglandin D synthase or the Epididymal Secretory Protein-1) between portions (Fig.2), clearly showing the proteins were included by the aliquots of cauda epididymal content being consequently emitted. The lack of detection of such particular proteins in a larger volume of ejaculate (such as the P2, Rodriguez-Martinez et al, 2009) suggests the presence of epididymal fluid is beneficial for cryosurvival. However, whether it is the presence of specific proteins, their concentration or of other factors (Saravia et al, 2009; 2010) remains to be explored.

Additionally, the proportion of live spermatozoa (SYBR+/PI-) after cryopreservation as well as boar sperm plasma membrane stability, assessed by Annexin-V and PI, and acrosome integrity (FITC PNA/PI) did not differ between portions of the SRF. It is well known that the freezing-thawing process results in a drastic reduction in the number of motile and viable spermatozoa (Eriksson and Rodríguez-Martínez, 2000), as confirmed in the present study. Furthermore, the surviving population shows a shorter life span and poor functionality with difficulties in reaching and penetrating the oocytes (Roca et al, 2006). Such impairment could be attributed to changes in the motility pattern, and/or altered plasma membrane structure and acrosome integrity during cryopreservation, which makes the spermatozoa more susceptible to capacitating factors, e.g. bicarbonate, calcium, present in the extender or in the female genital tract. In fact, Saacke and White (1972) found a significant positive relationship between the percentage of spermatozoa with intact acrosome, in fresh and frozen-thawed bull semen, and field fertility. In the present study, the freezing thawing procedure impaired motility, sperm membranes and acrosome integrity to the same extent in both SRF-portions, suggesting that spermatozoa present in these portions were equally sensitive/resilient to the cryopreservation stress.

In sum, the results of the present study showed an acceptable sperm cryosurvival in either portion of the SRF-ejaculate fraction (~ 47% total motility, ~ 55% live spermatozoa, ~ 51% spermatozoa with stable membrane, and ~ 53% spermatozoa with intact acrosome) yet suffering from inter-boar variation. The proteomic analysis done showed the portions of the SRF displayed very similar protein profiles, disregarding the concentrations of these proteins. These findings reinforce the hypothesis that, by still maintaining a relatively large proportion of epididymal fluid components, the seminal plasma from

either P1 or SRF-P1 is not deleterious to spermatozoa during freezing procedures, and therefore confirming they could be used for cryopreservation using a simplified protocol (Saravia et al. 2010). Freezing of only one portion of the SRF, either the P1 or any other part of the SRF would still allow for the use of the rest of the ejaculate to produce liquid semen, thus enabling comparisons of liquid semen fertility with froze-thawed fertility of the same ejaculate and boar. Fertility trials, using P1-derived AI doses, are being launched

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Tables

Table 1. Characteristics of two different portions (P1 and SRF-P1) of the sperm-rich fraction (SRF) of the boar ejaculate.

Male	P1		SRF-P1	
	Volume (mL)	Concentration (x10 ⁹ sptz/mL)	Volume (mL)	Concentration (x10 ⁹ sptz/mL)
A (n=5)	10.80±6.46 ^{aA}	1.46±0.26 ^{aA}	25.80±6.46 ^{aAB}	0.97±0.26 ^{aA}
B (n=5)	11.10±6.46 ^{aA}	2.85±0.26 ^{bB}	31.20±6.46 ^{aC}	1.89±0.26 ^{bA}
C (n=4)	10.68±7.22 ^{aAB}	1.65±0.29 ^{aA}	68.50±7.22 ^{bD}	1.25±0.29 ^{abAB}
D (n=5)	10.40±6.46 ^{aA}	1.45±0.26 ^{aA}	30.20±6.46 ^{aB}	0.91±0.26 ^{aA}
All (n=19)	10.74±0.25 ^A	1.86±0.20 ^A	37.37±5.73 ^B	1.25±0.14 ^B

Data are presented as mean ± standard error of the mean (SEM). ^{a,b}Values with different superscripts within a column indicate significant differences ($P<0.05$). ^{A,B,C,D}Values with different superscripts in the same row differ significantly ($P<0.05$).

Table 2. Motility parameters of fresh and frozen-thawed boar spermatozoa in two different portions of the ejaculated SRF (P1 and SRF-P1).

Fraction	Fresh semen			Post Thawed		
	Total motility (%)	Progressive motility (%)	Velocity (μ/s)	Total motility (%)	Progressive motility (%)	Velocity (μ/s)
P1 (n=19)	83.14±3.13	75.39±4.98	66.41±5.22	47.82±2.69	31.86±2.40	32.81±2.13
SRF-P1 (n=19)	89.10±0.56	85.15±1.70	64.61±4.52	47.23±3.68	32.19±3.26	32.74±2.74

Data are presented as mean ± standard error of the mean (SEM).

Table 3. Boar sperm plasma membrane integrity post-thaw, assessed by SYBR-14 and propidium iodide (PI) in two different portions of the ejaculated SRF (P1 and SRF-P1).

Fraction	SYBR+/PI- (%)	SYBR-/PI+ (%)	SYBR+/PI+ (%)
P1 (n=19)	54.82±2.92	41.67±2.77	3.51±0.38
SRF-P1 (n=19)	55.49±2.15	40.69±2.08	3.83±0.39

Data are presented as means ± standard error of the mean (SEM). SYBR+/PI- = Live spermatozoa; SYBR-/PI+ = Dead spermatozoa; SYBR+/PI+ = Dying spermatozoa.

Table 4. Boar sperm plasma membrane stability post-thaw, assessed by Annexin-V and propidium iodide (PI) in two different portions of the ejaculated SRF P1 and SRF-P1).

Fraction	An-PI+ (%)	An+PI+ (%)	An-PI- (%)	An+PI- (%)
P1 (n=19)	21.83±1.06	24.74±1.87	50.80±1.91	2.12±0.52
SRF-P1 (n=19)	20.93±1.24	23.55±1.56	51.67±1.63	3.24±0.88

Data are presented as means ± standard error of the mean (SEM). An-PI+ and An+PI+ = Cells with a damaged plasma membrane; An-PI- = Viable cells with stable plasma membrane; An+PI- = Cells with instable plasma membrane, but intacted.

Table 5. Acrosomal status and sperm viability of post-thaw spermatozoa from two different fractions of the ejaculate SRF (P1 and SRF-P1) as detected with FITC PNA/PI, H33342, using flow cytometry.

Fraction	Live unreacted (%)	Dead unreacted (%)	Dead reacted (%)	Live reacted (%)
P1 (<i>n</i> =19)	58.52±2.38	29.62±1.73	10.91±1.65	0.72±0.13
SRF-P1 (<i>n</i> =19)	58.23±2.14	29.09±1.46	11.24±1.22	1.13±0.16

Data are presented as means ± standard error of the mean (SEM).

Figures (including figure texts)

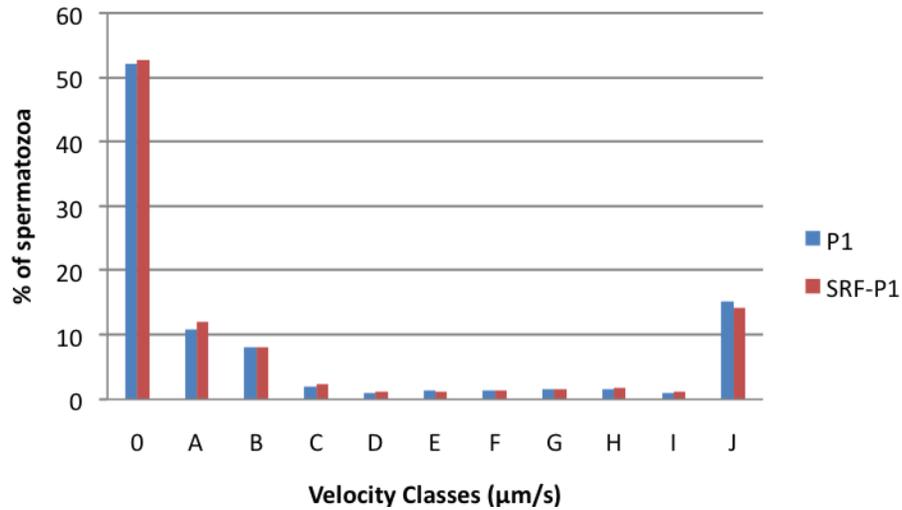


Figure 1. Speed classes of frozen-thawed boar spermatozoa in two different portions of the ejaculated SRF (P1 and SRF-P1). 0=0 $\mu\text{m/s}$ (Immotile); A=0-10 $\mu\text{m/s}$ (Non-progressive); B=10-35 $\mu\text{m/s}$; C=35-40 $\mu\text{m/s}$; D=40-45 $\mu\text{m/s}$; E=45-50 $\mu\text{m/s}$; F=50-55 $\mu\text{m/s}$; G=55-60 $\mu\text{m/s}$; H=60-70 $\mu\text{m/s}$; I=70-75 $\mu\text{m/s}$; J=>75 $\mu\text{m/s}$.

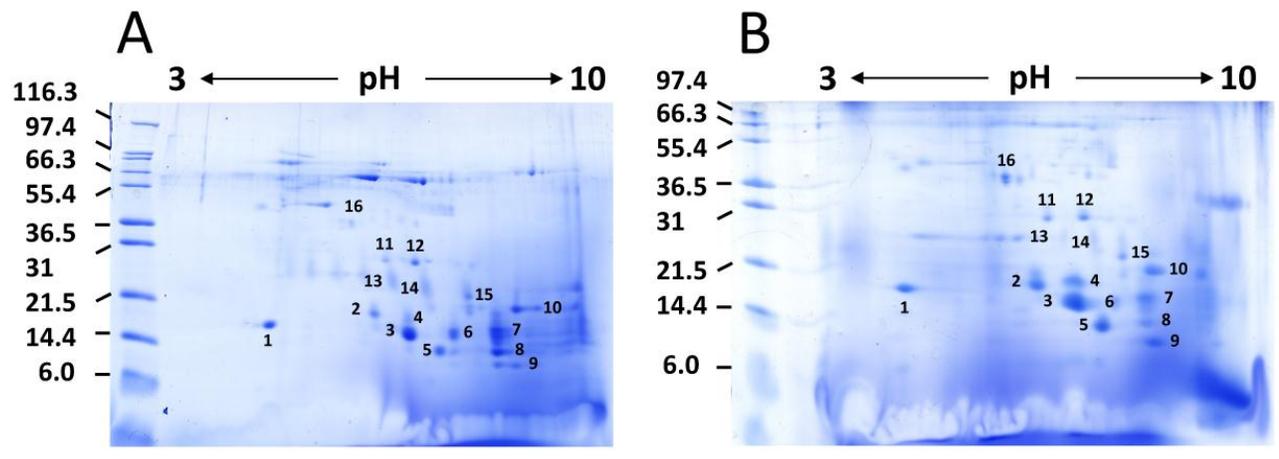


Figure 2. Comparison of the 2DE separations of the total proteins from the P1 (A) and SRF-P1 (B) portions of the SRF. Major protein spots shared between P1 and SRF-P1, as judged by pI, apparent molecular mass, identical tryptic peptide mass fingerprint, and CID-MS/MS analysis, are labeled with the same numbers, and were identified by MS/MS as lipocalin (spot 1); epididymal secretory protein-1 (spots 2 and 3); and spermadhesin PSP-I isoforms (spots 5-10).

Table 6. Mass spectrometric identification of 2DE-separated protein spots from P1 and SRF – P1. Numbers correspond to spots labelled in Fig.2. C, carbamidomethylated cysteine; X, Leu or Ileu. Ions used for identification of porcine prostaglandin D synthase in a 1D SDS-PAGE separation of P1 and SRF – P1 proteins are also listed.

Spot number	m/z	z	Amino acid sequence	Protein	Accession code
1	584.6	2	GTPXANGDXAXK	lipocalin-9	XP_001917526
	611.6	3	GAVDGQFSNAAXAQTDXR		
	559.9	2	NAWXQXFAR		
2, 3	784.4	2	DQTYSYLNKLPVK	Epididymal secretory protein-1	O97763
5-10	718.3	2	LTDDYGTIFTYK	Spermadhesin PSP-I	P35495
	524.8	2	LDYHACGGR		
	567.6	3	DSGHPASPYEIIIFLR		
	604.8	2	FCEGLSILNR		
SDS-PAGE	675.3	2	GFTEDGIVFLPR	Prostaglandin D synthase	Q765P8
	638.6	3	NYALLHTESGSPGPAFR		