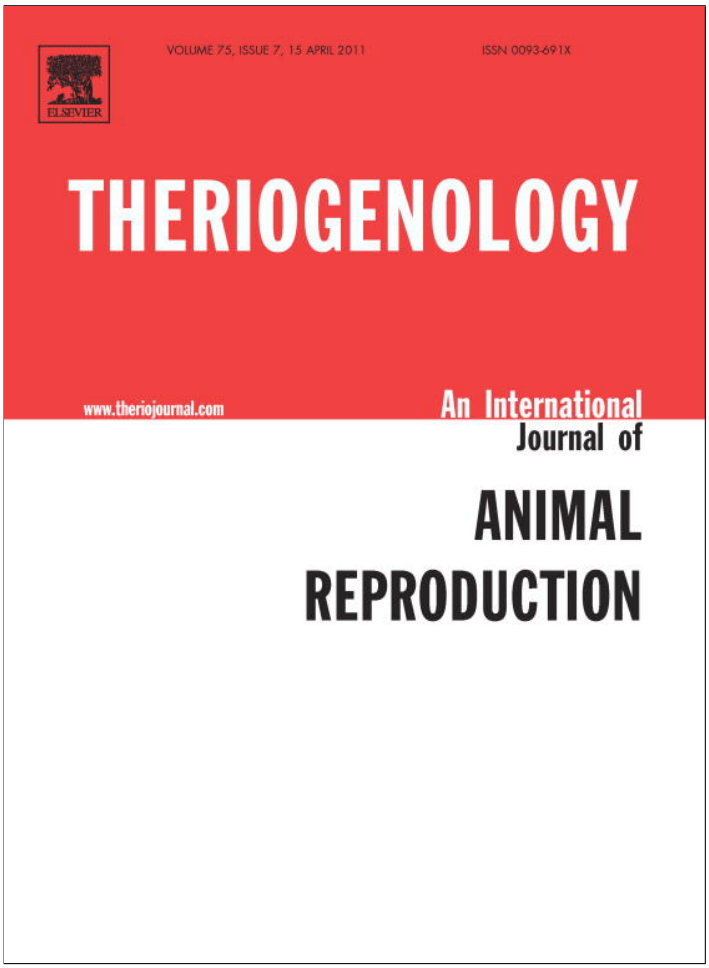

Available online at www.sciencedirect.com

ScienceDirect

Theriogenology 75 (2011) 1175–1184

Theriogenology

www.theriojournal.com

Quality of boar spermatozoa from the sperm-peak portion of the ejaculate after simplified freezing in MiniFlatpacks compared to the remaining spermatozoa of the sperm-rich fraction

A.P. Siqueira[a], M. Wallgren[a,b,*], M.S. Hossain[c], A. Johannisson[c], L. Sanz[d], J.J. Calvete[d], H. Rodríguez-Martínez[e]

[a] *Division of Reproduction, Swedish University of Agricultural Sciences (SLU), SE-75007, Uppsala, Sweden*
[b] *Quality Genetics, SE-24292, Hörby, Sweden*
[c] *Department of Anatomy, Physiology and Biochemistry, SLU, Uppsala, Sweden*
[d] *Instituto de Biomedicina de Valencia, CSIC, 46010 Valencia, Spain*
[e] *Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden*

Received 2 September 2010; received in revised form 29 October 2010; accepted 10 November 2010.

Abstract

Boar sperm viability post-thaw differs depending on the ejaculate fraction used, with spermatozoa present in the first 10 mL of the sperm-rich fraction (SRF) (portion 1, P1, sperm-peak portion) displaying the best cryosurvival *in vitro* compared with that of spermatozoa from the rest of the ejaculate (portion 2 of the SRF plus the post-spermatic fraction), even when using simplified freezing routines. This viability apparently relates to the specific profile of seminal plasma in P1 (i.e., glycoprotein and bicarbonate concentrations, and pH). However, spermatozoa from P1 have not been compared with spermatozoa from the rest of the SRF (SRF–P1, usually 30–40 mL of the SRF), which is routinely used for freezing. We compared P1 with SRF–P1 in terms of sperm kinematics (using the QualiSperm™ system), while membrane integrity (SYBR-14/PI), acrosome integrity (FITC PNA/PI), and sperm membrane stability (Annexin-V) were explored using flow cytometry. As well, total protein concentration and the proteomics of the seminal plasma (SP) of both portions of the SRF were studied using two-dimensional electrophoresis (2DE), mass fingerprinting (MALDI-TOF), and collision-induced dissociation tandem mass spectrometry (CID-MS/MS) on selected peptides. The SRF portions were collected weekly 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. Post-thaw sperm motility reached 50%, without differences between SRF portions, but with clear inter-boar variation. Neither plasma membrane nor acrosome integrity differed (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 SRF portions. While P1 and SRF–P1 clearly differed in relative total protein contents, as expected, they displayed very similar protein profiles as assessed using 2DE and mass spectrometry (tryptic peptide mass fingerprint analysis and CID-MS/MS), indicating a similar emission of epididymal protein content.

1. Introduction

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 the sperm-rich fraction (SRF), in which the bulk of spermatozoa is verted, particularly at the beginning (the so-called sperm-peak portion, or portion 1 (P1), a 10-mL portion whose epididymal caudal fluid content is high [1]). The rest of the SRF has high

* Corresponding author. Tel.: +46 18 672173; fax: +46 18 673545.
E-mail address: margareta.wallgren@kv.slu.se (M. Wallgren).

0093-691X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2010.11.024

numbers of spermatozoa, mixed with a richer protein secretion from the vesicular glands. The post-sperm-rich fraction (PSRF) contains decreasing sperm numbers and increasing secretions of the vesicular, prostate, and, by the end of the ejaculation, bulbourethral glands [2].

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 [1] and events preceding fertilization [3]. Thus it is not surprising that certain amounts of SP present in slightly cooled or frozen semen can improve sperm motility [4], maintain acrosome integrity [5], delay capacitation-like changes [5], and increase the resistance to cold shock [6,7] or oxidative stress [8,9]. Maintenance of these sperm attributes obviously leads to greater sperm longevity, cryosurvival, and, subsequently, fertility [10–13]. However, spermatozoa are not exposed to a mixture of SP fractions *in vivo*, as occurs when an ejaculate is collected [3], and it has been considered detrimental to sperm viability to store spermatozoa in SP [14]. Therefore, SP removal by extension and/or centrifugation has been customary during preservation. Yet, ~70% of variability in sperm cryosurvival can be attributed to male effects [15], as SP protein characteristics differ among boars [11,16].

Cryopreservation of boar semen has advanced over the past decade, with the inclusion of various additives in the semen extender [8,9,17,18], novel packaging systems [19,20], and simplified freezing protocols [21]. The latter were made possible by findings at SLU laboratory, demonstrating that spermatozoa from the sperm-peak portion of the ejaculate (P1) can equally survive conventional or simplified cryopreservation [20,21]. Simplified cryopreservation saves over 50% of the time required for conventional freezing, by avoiding extension or centrifugation. The spermatozoa present in P1 were superior at retaining viability *in vitro* compared with spermatozoa from the rest of the ejaculate (portion 2, second portion of the SRF plus the PSRF), displaying virtually no variation between boars [21]. Moreover, use of MiniFlatPacks™ (MFP) to pack and freeze P1 in single AI doses for deep intrauterine insemination [22] increased our ability to make more efficient use of the ejaculate [21].

Differences in the type and amounts of ions, proteins, bicarbonate, and pH, reminders of the milieu of the cauda epididymides, have been considered crucial for the better cryosurvival of P1 spermatozoa [2,23]. Cross-exposure of spermatozoa from either portion (P1 vs P2) to their native SP could clearly influence their cryosurvival, reinforcing the above concept: particular portions of the SP modulate sperm structure, intactness, and, ultimately, function [23]. Specifically, differences in SP proteomics have been described [3,23] and different SP protein profiles have been found between boars of different *in vivo* fertility [16]. 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, which primarily aiming to preserve the most spermatozoa without incurring in handling difficulties, the SRF is the fraction collected and used. The SP is routinely extended before cooling to +15 °C, and then usually removed several hours later by centrifugation. Although they have been compared with the rest of the ejaculate without removing the accompanying native SP [21], spermatozoa from P1 have not yet been compared with the rest of the spermatozoa in the SRF (SRF minus P1, usually 30–40 mL of the SRF) for possible differences in cryosurvival and SP protein composition when using simplified freezing.

The present experiment aimed to determine whether there were any differences in cryosurvival between spermatozoa in either portion, in terms of sperm kinematics (explored using the QualiSperm™ system), and in terms of membrane integrity (SYBR-14/PI), acrosome integrity (FITC PNA/PI), and sperm membrane stability (Annexin-V) (explored using flow cytometry—FC). Furthermore, 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) on selected peptides.

2. Material and methods

2.1. Animals

Four mature boars (1.5–4 y 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 [24], and provided with water *ad libitum*. The experimental protocol had previously been approved by the Ethics

Committee for Experimentation with Animals, Uppsala, Sweden.

2.2. Semen collection and primary processing

Ejaculates were collected once weekly for 4–5 consecutive weeks from each boar. Collection was done manually (gloved-hand technique) to enable partition of the SRF. The first 10 mL of the SRF (portion 1, P1) and the rest of the SFR (SRF–P1, where the part used was restricted to 30–40 mL) were collected into prewarmed 12- or 50-mL graduated plastic tubes, respectively. Only ejaculates with ≥70% motile spermatozoa and ≥80% morphologically normal spermatozoa were used. Sperm concentration was manually assessed using a Bürker chamber [25]. Total sperm numbers in P1 and SRF–P1 were calculated 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 (3000 × g / 20 min) with the SP supernatant moved to a clean tube before each subsequent centrifugation. After the last centrifugation, the SP harvested from the portions was separately filtered through disposable filters 0.2 μm in diameter (Filtropur S; Sarstedt AG & Co, Nümbrecht, Germany), put into 10-mL 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 the "simplified freezing method" protocol for MiniFlatPacks (MFPs) [21]. In brief, spermatozoa from P1 or SRF–P1 were kept in their own SP in the dark at room temperature (+20–22 °C) for 30 min. Thereafter, the semen was mixed with a lactose–egg yolk (LEY) extender [20] at a ratio of one to two parts of semen to one part of extender, and cooled to 5 °C within 1.5 h. The LEY-extended 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 approximately 1.0×10^9 spermatozoa/mL. The LEYGO was composed of LEY, Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA), and glycerol (3% final proportion) [23]. Spermatozoa were then transferred to a cool cabinet at 5 °C (IMV, L'Aigle, France) and packaged in MFPs, each holding ~500×10^6 spermatozoa. The MFPs were heat-sealed and cooled/frozen using a programmable freezer (Mini Digitcool 1400; IMV, L'Aigle, France) at a rate of 3 °C/min from 5 to -5 °C; 1 min was allowed for crystallization, and thereafter the MFPs were cooled at 50 °C/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 20 s.

2.5. Sperm analyses

2.5.1. Sperm motility

Sperm motility was evaluated on fresh and frozen–thawed semen. Three motility parameters, i.e., total motility (%), progressive motility (%), and sperm velocity (μm/s), were determined using the QualiSperm™ system, version 1.3 (Biophos, Pfäffikon, Switzerland) [26]. After thawing, the semen suspension was extended with BTS + LEY (95 mL of BTS + 5 mL of LEY) at a 1:5 to 1:10 ratio, to give a sperm concentration of approximately 40–50 × 10^6 spermatozoa/mL, and incubated at 38 °C for 30 min. Following gentle mixing, 5 μL of each sample was placed in a prewarmed (38 °C) 10-μm-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 100× magnification. The spermatozoa were recorded in one field at a rate of 50 frames/s (200 frames in total) using an MVD640-48-U2-10 Photonfocus camera (Photonfocus AG, Lachen, Switzerland). The data recorded were the proportions of motile, progressive motile, and immotile spermatozoa, and their mean speed. In addition, classes of sperm velocity (μm/s) in post-thawed samples were recorded. Spermatozoa were distributed (%) in 11 velocity classes: 0 = 0 μm/s (Immotile); A = 0–10 μm/s (Non-progressive); B = 10–35 μm/s; C = 35–40 μm/s; D = 40–45 μm/s; E = 45–50 μm/s; F = 50–55 μm/s; G = 55–60 μm/s; H = 60–70 μm/s; I = 70–75 μm/s; and J ≥ 75 μm/s.

2.5.2. Sperm plasma membrane integrity and stability, and acrosome integrity

These measurements were made using an LSR FC (Becton Dickinson, San José, CA, USA) following established protocols as described in Saravia et al [27]. 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 μL of semen to 500 μL of BTS). The suspension was centrifuged (10 min, 25 °C, 300 × g) and the pellet resuspended in 1 mL of BTS. The sperm suspension was loaded with 1 μL of SYBR-14 (1:50)

and 5 μL of 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 were classified as live (SYBR+/PI-), dying (SYBR+/PI+), or dead (SYBR-/PI+) (as described in Saravia et al [20]). Acrosome intactness was assessed using 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 μL), Ho3342 (1 μL), and FITC PNA (5 μL), incubated for 10 min at 37 °C, and analyzed as described in Spjuth et al [28]. Viable spermatozoa (PI-negative, PI-) with an intact outer acrosome membrane deemed PNA-FITC-positive (PNA-FITC+) were judged as acrosome-reacted, while PNA-FITC-negative (PNA-FITC−) spermatozoa were considered having an intact outer acrosome membrane. Sperm membrane architecture and early destabilization were evaluated by probing the spermatozoa with Annexin-V/PI as described by Peña et al [17] and modified by Saravia et al [23] using Annexin-V-FITC apoptosis detection kits I and II (Pharmigen, San Diego, CA, USA). Frozen semen samples were thawed and an aliquot of 10 μL was extended with 90 μL of binding buffer. To this suspension, 90 μL of binding buffer, 3.5 μL of Annexin, 10 μL of PI, and 2 μL of Ho342 (500 μg/mL) were added before incubation in darkness at room temperature for 15 min. An additional 400 μL 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 spectrophotometry, using bovine serum albumin (BSA, 1 mg/mL) to plot a standard curve, and readings at 595-nm absorbance. A linear regression equation was used to determine the total protein concentration in the SP samples.

2.6.2. Gel electrophoresis of seminal plasma

The SP protein profile of each sample was determined using two-dimensional electrophoresis (2DE). To run the first dimension (isoelectric focusing), 7-cm-long IPG strips (Bio-Rad Laboratories, Hercules, CA, USA), pH range 3–10, were rehydrated in 150 μL of protein solution (containing a total of approximately 200 mg of protein) for 4 h at room temperature. Isoelectric focusing (IEF) was carried out using a Protean IEF Cell (Bio-Rad) with the following conditions: 1 h at 500V, 2.5 h at 1000V, and 2.5 h at 5000V. After focusing, the strips were equilibrated in 50 mM Tris-HCl, 6 M 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 Brilliant Blue solution (EasyStain, Invitrogen, Carlsbad, Ca, USA) and destained in deionized water.

2.6.3. 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, alkylation with iodoacetamide, and in-gel digestion with sequencing-grade bovine pancreas trypsin (Roche, San Cugart del Vallés, Barcelona, Spain) using a ProGest digestor (Genomic Solutions, Cambridgeshire, UK) following the manufacturer's instructions. Approximately 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 a-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% TFA, dried, and analyzed using a Voyager-DE Pro MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA), 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).

2.6.4. 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, Odense, Denmark). Doubly- or triply-charged

Table 1
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).

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, 8V; 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 the mean values for sperm parameters (using arcsine transformation in which data did not display 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 (i.e., fresh or post-thawing), and portion within the SRF fraction and their interaction. When overall significance was found, SNK (Student-Newman-Keul-Test) was used to compare more than two groups (ANOVA Post-hoc) and Student′s *t*-test to compare two groups. The results are presented as means ± SEM and the level of significance was set at $P < 0.05$. A correlation analysis (Spearman) was carried out between pre-freezing variables (excluding those derived from the subpopulation study) and post-thaw variables.

3. Results

P1 had a mean sperm concentration of 1.86 ± 0.20 × 10^9 spermatozoa/mL (±SEM, n = 19), which significantly differed from SRF–P1 where the mean concentration was 1.25 ± 0.14 × 10^9 spermatozoa/mL, ($P < 0.05$). The concentration within both SRF portions differed among boars ($P < 0.01$).

Motility parameters in both SRF portions (P1 and SRF–P1) for fresh and frozen–thawed samples are presented in Table 1. There were no differences between portions for any parameter analyzed in fresh semen, but there was variations among boars for total motility (82.8 ± 3.7% up to 90.6 ± 4.7%, $P < 0.05$).

Post-thaw, total sperm motility, progressive motility, and velocity reached means (±SEM) of 47.82 ± 2.69%, 31.86 ± 2.40%, and 32.81 ± 2.13 μ/s for P1 and 47.23 ± 3.68%, 32.19 ± 3.26%, and 32.74 ± 2.74 μ/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 displayed the best results with regards to total post-thaw motility (P1, 54.75 ± 3.63%; SRF–P1, 59.78 ± 3.63%), while boar B displayed the worst results (P1, 32.2 ± 3.6; SRF–P1, 25.6 ± 1.8).

The speed sperm classification did not reveal any differences between SRF portions ($P > 0.05$), Among motile spermatozoa most of them were seen in speed classes A, B and J (A = non progressive, B = 10–35μm/s, J = 70–75 μm/s). However the speed classification did reveal differences among animals, particularly between boars B and D for all classes investigated ($P < 0.05$). Boar B had the lowest percentage of sper-

Table 2
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 3
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.

matozoa (6.5 ± 1.3%) in the rapid progressive class (K), whereas boar D had the highest percentage of spermatozoa (23.09 ± 1.2%) in the rapid progressive class, 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-thaw parameters.

The proportions of spermatozoa with an intact plasma membrane post-thaw, as assessed by SYBR-14/PI, are presented in Table 2. 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 ± 1.63%). The data on the assessment of early sperm plasma membrane destabilization after thawing, using Annexin-V and PI, are presented in Table 3. Post-thaw acrosome integrity is presented in Table 4. Proportions of live-acrosome intact spermatozoa were 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, with the worst sperm kinematic values post-thaw, had the best results in relation to sperm plasma membrane integrity (63.49 ± 2.74%), stability (56.39 ± 2.01%), and acrosome integrity (63.35 ± 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. 1) 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 (Table 5). The major proteins shared between these fractions are labelled with same numbers in the panels of Fig. 1.

4. Discussion

The development of a commercially acceptable procedure to cryopreserve boar semen has been pursued. Specifically, the sought-after method would produce a unique small-volume AI dose with high sperm concentration and high cryosurvival, faster than at present, and thus allowing for coprocessing under routine conditions of commercial liquid semen production. Provided fertility can be maintained using deep-intra-uterine AI [29], the procedure would be in the best interest of the swine industry.

Use of a simplified-short freezing protocol [21] in combination with a well-defined portion of the boar ejaculate, for example, 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 semen handling from 8–9 h to 3–4 h. It also diminished inter-boar freezing variability, probably due to a combination of the SP com-

Table 4
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 (n = 19)	58.52 ± 2.38	29.62 ± 1.73	10.91 ± 1.65	0.72 ± 0.13
SRF-P1 (n = 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).

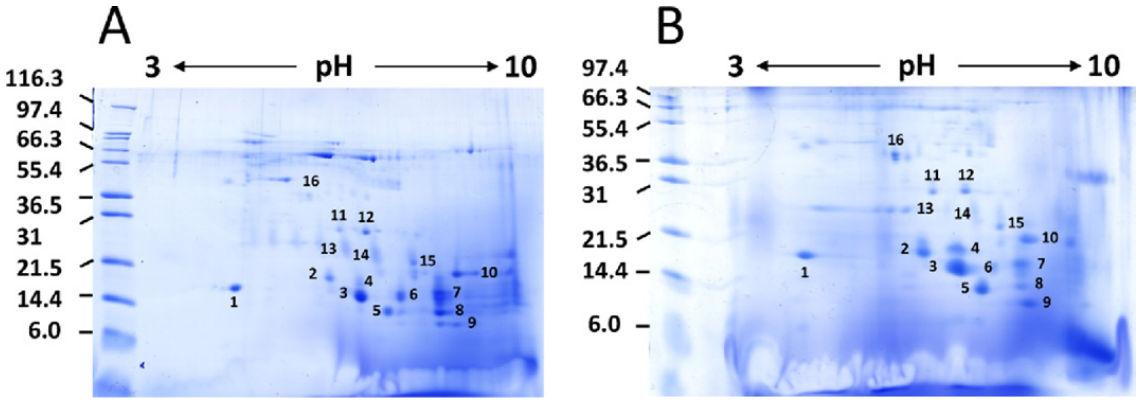

Fig. 1. Comparison of the 2DE separations of the total proteins from the P1 (A) and SRF–P1 (B) portions of the SRF. Major protein spots shared between P1 and SRF–P1, as judged by pI, apparent molecular mass, identical tryptic peptide mass fingerprint, and CID-MS/MS analysis, are labelled with the same 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).

position of P1 and the packing in cryobiologically advantageous containers such as the MFP [23], and allowed the use of the rest of the ejaculate to produce routine liquid semen doses for conventional AI [21].

The present study compared two portions of the SRF (P1 vs SRF–P1), using the same method for simplified cryopreservation as in Saravia et al [21], to determine whether either one could be used. The sperm concentration in P1 represented the sperm peak, containing almost 30% of the total number of spermatozoa constituting the SRF, thus confirming previous data for other boars [21]. In addition, the potential number of doses (of 1×10^9 spermatozoa/mL) from each fraction was 19 for P1 and 46 for SRF–P1, reinforcing the idea that the use of the P1 portion processed using this newly simplified freezing protocol produces enough doses to allow the swine industry to build genetic banking without compromising commercial boar stud routines [21].

Sperm kinematics (i.e., % 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 from 30 to 60% of total motile spermatozoa, but without differences when SRF portions were compared. Male B spermatozoa displayed the poorest post-thaw motility of both portions studied. Therefore, an aliquot of each SRF portion of this male was thawed and incubated in the presence of 5 mM caffeine (Sigma Chemical Co., St. Louis, MO, USA). The addition of caffeine significantly ($P < 0.05$) increased total motility (P1, 32.2 ± 3.6 vs 67.9 ± 0.6%; SRF–P1, 25.6 ± 1.8 vs 61.0 ± 4.2%), as well as progressive motility (P1, 19.1 ± 2.2 vs 55.5 ± 0.7%; SRF–P1, 15.9 ± 1.7 vs 45.9 ± 3.3%) and mean sperm velocity (P1, 27.6 ± 2.2 vs 61.2 ± 2.9 μm/s; SRF–P1, 25.5 ± 1.9 vs 43.4 ± 3.1 μm/s) in both SRF portions (ns).

Differences in post-thaw sperm quality between boars were sometimes found [30] and sometimes not [27]. Inter-animal differences in ability to sustain sperm

Table 5
Mass spectrometric identification of 2DE-separated protein spots from P1 and SRF–P1. Numbers correspond to spots labelled in Figure 2C, 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	584.62	GTPXANGDXAXK	lipocalin-9	XP_001917526
	611.63	GAVDGQFSNAAXAQTDXR		
	559.92	NAWXQXFAR		
2,3	784.42	DQTYSYLNKLPVK	Epididymal secretory protein-1	O97763
5–10	718.32	LTDDYGTIFTYK	Spermadhesin PSP-I	P35495
	524.82	LDYHACGGR		
	567.63	DSGHPASPYEIIFLR		
	604.82	FCEGLSILNR		
SDS page	675.32	GFTEDGIVFLPR	Prostaglandin D synthase	Q765P8
	638.63	NYALLHTESGPSPGPAFR		

cryopreservation have been associated with SP composition [31,32], genetic factors [33], and the existence of distinct spermatozoa subpopulations in the ejaculate, in terms of morphological characteristics [34] and movement patterns [35].

In this context, we ranked the various 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, whose sperm displayed the poorest post-thaw motility, also produced the fewest spermatozoa with rapid progressive motility (>75 μm/s). There is obviously a need to test a larger number of boars, correlating the different speed sperm populations with 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 [36].

Previous results confirmed the existence of essential differences between P1 and the rest of the ejaculate [1,23] in terms of types and amounts of ions, proteins, bicarbonate, and pH, indicating that 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 clearly indicate that, despite SRF–P1 having a significantly larger relative content of total protein than does the P1, these portions of the SRF displayed very similar protein profiles as assessed using 2DE and mass spectrometry (tryptic peptide mass fingerprint analysis and CID-MS/MS) (Fig. 1). Of particular interest were the similar contents of epididymal-contained proteins (such as the lipocalin-type prostaglandin D synthase or the epididymal secretory protein-1) between portions (Fig. 1), clearly indicating that the proteins were included in the aliquots of cauda epididymal content subsequently emitted. The apparent lack of these particular proteins in a larger volume of ejaculate (such as the P2) [1] suggests that the presence of epididymal fluid is beneficial for cryosurvival. However, whether it is the presence of specific proteins, their concentrations, or other factors [21,23] that are involved, remains to be explored.

In addition, the proportion of live spermatozoa (SYBR+/PI-) after cryopreservation and boar sperm plasma membrane stability (assessed using 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 drastically reduces the number of motile and viable spermatozoa [19], as confirmed in the present study. Furthermore, the surviving population has a shorter life span and poor functionality with difficulties in reaching and penetrating the oocytes [37]. 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, such as bicarbonate and calcium, present in the extender or in the female genital tract [1,3,4,38]. 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 indicated acceptable sperm cryosurvival in both portions of the SRF ejaculate fraction (~47% total motility, ~55% live spermatozoa, ~51% spermatozoa with stable membrane, and ~53% spermatozoa with intact acrosome), though inter-boar variation was evident. The proteomic analysis indicated that the SRF portions displayed very similar protein profiles, disregarding the concentrations of these proteins. These findings reinforce the hypothesis that, by 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 it could be used for cryopreservation using a simplified protocol [21]. Freezing only one portion of the SRF, either the P1 or any other part of the SRF, would still allow the rest of the ejaculate to be used to produce liquid semen, thus enabling comparisons of liquid semen fertility with frozen–thawed fertility of the same ejaculate and boar. Fertility trials, using P1-derived AI doses, are being launched.

Acknowledgments

This study was supported by the Swedish Farmer's Foundation for Agricultural Research (SLF), Stockholm, and grants BFU2007-61563 and BFU2010-17373 from the Ministerio de Ciencia e Innovación, Madrid, Spain. Scholarships from the Swedish Institute (SI), Stockholm, to APS and Md-H are gratefully acknowledged. The authors thank Karin Selin-Wretling and Annika Rikberg for their technical assistance. Bio-

phos is acknowledged for letting us use the Quali-Sperm™ instrument.

References

[1] Rodriguez-Martinez H, Kvist U, Saravia F, Wallgren M, Johannisson A, Sanz L et al. The physiological roles of the boar ejaculate. In: Rodriguez-Martinez, H. Vallet and J.L. Ziecik, editors, A.J. Control of Pig Reproduction, vol. VIII. Nottingham University Press: Nottingham, UK, 2009, p.1–21.

[2] Mann T, Lutwak-Mann C. Male reproductive function and semen, 1st ed, New York: Springer-Verlag Berlin Heidelberg, 1981.

[3] Rodríguez-Martínez H, Saravia F, Wallgren M, Tienthai P, Johannison A, Vázquez JM, et al. Boar spermatozoa in the oviduct. Theriogenology 2005;63:514–35.

[4] Rodriguez-Martinez H. Aspects of the electrolytic composition of boar epididymal fluid with reference to sperm maturation and storage. Reprod Domest Anim 1991;Suppl 1:13–27.

[5] Maxwell WM, de Graaf SP, Ghaoui Rel-H, Evans G. Seminal plasma effects on sperm handling and female fertility. Soc Reprod Fertil Suppl 2007;64:13–38.

[6] Pursel VG, Johnson LA, Schulman, LL. Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. J Anim Sci 1973;37:528–31.

[7] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60–61:481–92.

[8] Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004;25:397–405.

[9] Roca J, Rodríguez MJ, Gil AG, Carvajal G, Garcia EM, Cuello C, Vazquez JM, Martinez EA. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005;26:15–24.

[10] Zhu J, Xu X, Cosgrove JR, Foxcroft GR. Effects of semen plasma from different fractions of individual ejaculates on IVF pigs. Theriogenology 2000;54:1443–52.

[11] Hernández M, Roca J, Calvete JJ, Sanz L, Muiño-Blanco T, Cebrián-Pérez JA et al. Cryosurvival and in vitro fertilizing capacity after thaw is improved when boar spermatozoa are frozen in the presence of seminal plasma from good freezer boars. J Androl 2007;28:689–97.

[12] Okazaki T, Abe S, Yoshida S, Shimada M. Seminal plasma damages sperm during cryopreservation, but its presence during thawing improves semen quality and conception rates in boars with poor post-thaw semen quality. Theriogenology 2009;71:491–8.

[13] Garcia JC, Dominguez JC, Peña FJ, Alegre B, Gonzalez R, Castro MJ, Habing GG, Kirkwood RN. Thawing boar semen in the presence of seminal plasma: Effects on sperm quality and fertility. Anim Reprod Sci 2010;119:160–65.

[14] Kawano N, Shimada M, Terada T. Motility and penetration competence of frozen–thawed miniature pig spermatozoa are substantially altered by exposure to seminal plasma before freezing. Theriogenology 2004;61:351–64.

[15] Roca J, Hernández M, Carvajal G, Vázquez JM, Martínez EA. Factors influencing boar sperm cryosurvival. J Anim Sci 2006;84:2692–99.

[16] Flowers WL, Turner ZA. Relationships between seminal plasma protein profiles and estimates of fertility for boars. In: VIth International Conference on Pig Reproduction. Greenville, Missouri USA 2001;I:65.

[17] Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Assessment of fresh and frozen–thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 2003;60:677–89.

[18] Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. Zygote 2004;12:117–24.

[19] Eriksson BM, Rodríguez-Martínez H. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. Anim Reprod Sci2000;63:205–20.

[20] Saravia F, Wallgren M, Nagy S, Johannisson A, Rodríguez-Martínez H. Deep freezing of concentrated boar semen for intrauterine insemination: effects on sperm viability. Theriogenology 2005;63:1320–33.

[21] Saravia F, Wallgren M, Rodríguez-Martínez H. Freezing of boar semen can be simplified by handling a specific portion of the ejaculate with a shorter procedure and MiniFlatPack packaging. Anim Reprod Sci 2010;117:279–87.

[22] Wongtawan T, Saravia F, Wallgren M, Caballero I, Rodríguez-Martínez H. Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. Theriogenology 2006;65:773–87.

[23] Saravia F, Wallgren M, Johannisson A, Calvete JJ, Sanz L, Peña FJ, Roca, J, Rodríguez-Martínez H. Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. Theriogenology 2009;71:662–75.

[24] Simonsson A. Näringsrekommendationer och fodermedelstabeller till svin (Nutritional recommendations and feedstuff for swine). Swedish University of Agricultural Sciences, Uppsala, 1994, p. 71.

[25] Bane A. A study on the technique of hemocytometric determination of sperm motility and sperm concentration in bull semen. Cornell Vet 1952;42:518–31.

[26] Tejerina F, Buranaamunuay K, Saravia F, Wallgren M, Rodriguez-Martinez H. Assesment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments. Theriogenology 2008;69:1129–38.

[27] Saravia F, Hernández M, Wallgren M, Johannisson A, Rodríguez-Martínez H. Controlled cooling during semen cryopreservation does not induce capacitation of spermatozoa from two portions of the boar ejaculate. Int J Androl 2007;30:485–99.

[28] Spjuth L, Johannisson A, Lundeheim N, Rodríguez-Martínez H. Early pre-pubertal exposure to low-dose oral di(2-ethylhexyl) phthalate does not affect sperm plasma membrane stability, acrosomal integrity or chromatin structure in the post-pubertal boar. Theriogenology 2007;68:186–95.

[29] Martinez EA, Vazquez JM, Roca J, Lucas X, Gil MA, Parilla I, Vasquez JL, Day B. Reproduction 2001;122:289–96.

[30] Peña FJ, Saravia F, Núnez-Martínez I, Johannisson A, Wallgren M, Rodríguez-Martínez H. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? Animal Reprod Sci 2006;93:101–13.

[31] Flowers WL. Relationship between seminal plasma proteins and boar fertility. Swine News (USA) 2001;I:1–4.

[32] Novak S, Ruiz-Sánchez A, Dixon WT, Foxcroft GR, Dyck MK. Seminal plasma proteins as potential markers of relative fertility in boars. J Androl 2010;31:188–200.

[33] Thurston LM, Watson PF, Holt WV. Semen cryopreservation: A genetic explanation for species and individual variation? Cryoletters 2002;23:255–62.

[34] Thurston LM, Watson PF, Mileham, AJ, Holt WV. Morphologically distinct sperm subpopulations defined by fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl 2001;22:382–94.

[35] Cremades T, Roca J, Rodriguez-Martinez H, Abaigar T, Vazquez JM, Martinez EA. Kinematic changes during the cryopreservation of boar spermatozoa. J Androl 2005;26:610–8.

[36] Rodriguez-Martinez H. State of the art in farm animal sperm evaluation. Reprod Fert Dev 2007;19:91–101.

[37] Roca J, Rodriguez-Martinez H, Vazquez JM, Bolarín A, Hernandez M, Saravia F, Wallgren M, Martínez EA. Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination. In: Ashworth CJ and Kraeling RR, editors. Control of Pig Reproduction VII. Nottingham Univ. Press, Manor Farm: Thrumpton, UK, 2006, p. 261–275 (Soc Reprod Fert Suppl 62).

[38] Rodriguez-Martinez H, Tienthai P, Suzuki H, Funahashi, Ekwall H, Johannisson A. Oviduct involvement in sperm capacitation and oocyte development. Control of Pig Reproduction VI. 2001;129–45 (Reproduction Suppl 58).