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ScienceDirect
Theriogenology 76 (2011) 436–447
Theriogenology
www.theriojournal.com

Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage

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Received 28 April 2010; received in revised form 16 February 2011; accepted 16 February 2011

Abstract

Whole seminal plasma (SP) enhances the function and fertility of frozen/thawed ram sperm. The objective of the current study was to investigate whether SP proteins capable of binding to molecules from the sperm plasma membrane were conserved among ram breeds, and whether these proteins were sufficient to overcome cryopreservation-induced reductions in sperm quality. Whole ram SP, obtained from rams of various breeds, improved progressive motility of frozen/thawed sperm at all times evaluated ($P < 0.05$); however, it did not improve total motility (15 min, $P = 0.480$; 30 min, $P = 0.764$; and 45 min, $P = 0.795$). To identify SP proteins responsible for this effect, a new method was developed to retain SP proteins that bound specifically to the sperm membrane by immobilization of sperm membrane proteins. These proteins specifically bound to the sperm surface, especially the acrosomal region. Lactotransferrin, epididymal secretory protein E1, Synaptosomal-associated protein 29, and RSVP-20 were identified (mass spectrometry) in this fraction. The retained SP proteins fraction repaired ultrastructural damage of frozen/thawed sperm and, with the addition of fructose, significantly improved motility of frozen/thawed sperm. We concluded that SP proteins that bound to the sperm membrane were conserved among ram breeds, and that when added to frozen/thawed semen (along with an energy source), they repaired ram sperm damage and enhanced sperm motility.

Keywords: Sperm; Seminal plasma proteins; Ram; Semen cryopreservation

1. Introduction

Mammalian seminal plasma (SP), a physiological secretion from glands of the male reproductive tract, has important roles in sperm maturation, and functions as a vehicle for ejaculated sperm [1,2]. It is widely known that cryopreservation, which involves cooling, freezing and thawing, is deleterious to sperm function [3]. Sperm cryoinjury includes premature induction of a capacitation-like status, so-called cryocapacitation [4–6]. However, when SP was added after semen thawing, it increased resistance of ram sperm to cold shock [7,8] and the deleterious effects of cryopreservation [9,10]. The addition of SP to frozen-thawed ram sperm improved motility, viability, acrosome integrity, and mitochondrial respiration [11–13]. These beneficial effects were attributed to SP proteins [8,11,14–16], especially RSVP14 and RSVP20 [7,8], produced in the

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doi:10.1016/j.theriogenology.2011.02.020

seminal vesicles. These proteins were purified (exclusion chromatography) from a SP fraction of Rasa Aragonesa rams; they attached to the entire sperm surface, especially the acrosomal region, and appeared to act by stabilizing the sperm membrane and participating in capacitation [7,8]. In SP from Frisian rams, we previously found these two proteins among five proteins that attached to sperm after incubation with SP [14]. Therefore, we inferred that SP proteins that attach to the sperm surface may be conserved among breeds. The protective effect of these proteins might be due to membrane stabilization acting as decapacitating factors, and later acting as membrane modifiers during capacitation [2]. Moreover, since RSVP14 and RSPV20 have antioxidant capacity [17] and cryocapacitation is partially induced by ROS production [6], the beneficial effect of these proteins might be related to protection against oxidative stress and premature capacitation [2].

Knowledge regarding SP molecules that attach to the surface of frozen/thawed sperm contributes to understanding molecular mechanisms of capacitation. In addition, identification of molecules responsible for the ability of SP to prevent and repair membrane damage could be used to improve post-thaw sperm quality and therefore, be applicable to sperm sorting [16]. The objective of the current study was to investigate whether SP proteins capable of binding to molecules from the sperm plasma membrane were conserved among ram breeds, and whether these proteins were able to overcome cryopreservation-induced reductions in sperm quality.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.1. Collection, freezing and thawing of ram sperm

Semen was collected (with an artificial vagina) from three mature *Ost-Friesisches Milchschaf* (Frisian) rams during the autumn (May, 2005). Ejaculates were maintained at 32 °C, and those with good wave motion ($\geq$ 4; range, 0 to 5), $>$ 80% progressively motile sperm, and $> 2.5 \times 10^9$ sperm/mL, were selected. For cryopreservation, at least six sessions of semen extraction of one ejaculate per animal were performed. Ejaculates from all the animals were pooled and semen was diluted (1×10^9 sperm/mL) in an extender containing 300 mM Tris, 37.7 mM citric acid, 94.7 mM glucose, 20% (v/v) egg yolk, 5% (v/v) glycerol, sodium G penicillin (1×10^6 IU/L), and streptomycin sulphate (1 g/L). Diluted semen was cooled to 5 °C over 2 h, held at that temperature for another 2 h, and 150 μL drops of diluted semen were dispensed in concavities on dry ice. After 10 min, the frozen pellets were plunged in liquid N_2 and stored until thawing.

For epididymal sperm, testes were obtained (castration) from two adult Frisian rams. The epididymides were immediately submitted to the laboratory, washed with warm PBS, and dissected with sterile scalpels. Sperm were collected (in 35 mm Petri dishes) in PBS (37 °C), by performing several incisions in the *cauda* epididymis with a surgical blade. Fresh epididymal and ejaculated sperm were harvested by centrifugation (100 $\times$ g, 10 min), washed twice with PBS, and membrane proteins were immediately prepared as described below.

Frozen sperm pellets were thawed in 200 μL of PBS in a water bath at 37 °C. To obtain sperm membrane proteins, thawed sperm were washed twice in PBS (100 $\times$ g, 10 min) and suspended in 1 mL PBS. To assess the SP repairing effect, a motile sperm population was obtained, then frozen-thawed semen was centrifuged (700 $\times$ g, 10 min) at room temperature in a Percoll® discontinuous gradient in PBS (90-60-30%) [14]. The pellet (200 μL) was collected and suspended in PBS (concentration, 5×10^6 sperm/mL). Initial total motility of all samples was $>$ 60%.

2.2. Collection of seminal plasma

Semen was collected from Frisian (three animals), Texel (two animals) and Corriedale (five animals) rams during autumn (May, 2005). At least six sessions of semen extraction of one ejaculate per animal were performed. To separate SP, semen was centrifuged (2 000 $\times$ g, 15 min, 4 °C) and supernatants were centrifuged again for SP collection. Finally, supernatants from all rams of the same breed were pooled (equal proportion from each ram), filtered through a 0.22 μm nitrocellulose acetate membrane (Microclar®, Tigre, BA, Argentina), and stored at −20 °C until used. Seminal plasma was thawed at room temperature. Based on protein concentrations of both fractions of filtrate, no protein was lost during filtration.

2.3. Solubilization and immobilization of sperm membrane proteins

Membrane proteins were obtained from fresh (ejaculated and epididymal) and frozen sperm. Semen pellets were thawed at room temperature and washed twice in PBS (100 $\times$ g, 10 min). Both fresh and thawed

sperm were suspended in 1 mL PBS and the concentration determined. Sperm suspensions were sonicated five times (30 s) at 60 Hz (Cole Parmer Instrument Co., Chicago, IL) and then centrifuged for 20 min at 12 000 × g at 4 °C. Finally, the supernatant was ultracentrifuged (120 000 × g, 3 h, 5 °C) and the membrane fraction was harvested from the pellet. The procedure was repeated twice to ensure isolation of the membrane fraction. Then, the pellet was suspended in 500 μL buffer TEN (10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 10 mM NaCl) containing 1 mM sodium ortovanadate, 1 mM sodium fluoride, 1 mM PMSF (phenylmethylsulfonyl fluoride), 10 μg/μL Pepstatin A, 0.1 mM TLCK (N^{α}-Tosyl-Lys-chloromethylketone, hydrochloride), and 0.5% Triton X-100. The suspension was incubated for 1 h at 4 °C and centrifuged (16 000 × g, 20 min, 4 °C). Solubilized sperm membrane proteins were washed with four volumes of PBS, concentrated through YM-10 membranes (Amicon), and stored at −20 °C until used. To assess the quality of the membrane fraction, preparations were tested by Western blotting using an anti-pan cadherin (Sigma C3678) as a membrane molecular marker. To evaluate the presence of lipids in the protein fraction, cholesterol was measured by an enzymatic method (Colestat, Wiener Laboratory, Rosario, Argentina). A drop of the concentrated solution (100 μg protein) was loaded onto 0.25 cm^2 PVDF membrane squares previously activated with 100% methanol for 5 min, and equilibrated on TBS (10 mM Tris pH 7.5 and 0.15 M NaCl) with five changes of buffer. The presence of proteins was monitored with Ponceau S staining. Membranes were blocked with 0.5% polyvinylpyrrolidone K-30 (PVP K-30) in TBS for 3 h. The entire assay was performed at room temperature.

2.4. Seminal plasma fractions

In all experiments, whole SP was obtained as described (Section 2.2.) or fractioned as described below.

2.4.1. Seminal plasma fraction with affinity for the sperm membrane

This fraction was obtained by incubating SP (500 μg proteins) with immobilized sperm membrane proteins prepared (as described in Section 2.3.) from frozen or fresh (epididymal or ejaculated) sperm in 200 μL TBS, overnight at room temperature, under end-to-end agitation to ensure interaction. Control immobilized sperm membrane proteins were incubated with TBS (under the same conditions). After incubation with SP, squares of PVDF containing immobilized sperm proteins were washed twice with 200 μL TBS to separate non-retained proteins (which were considered non-interacting SP proteins). Then, retained proteins were eluted twice with 200 μL buffer 10 mM glycine-HCl pH 2.2 and 1.5 M NaCl, and neutralized with 5 μL Tris-HCl 2 M pH 9.5. This fraction was designated interacting SP proteins.

2.4.2. Seminal plasma depleted of proteins with affinity for the sperm membrane

This was obtained by incubating SP (2500 μg protein) with immobilized sperm membrane proteins (as described in 2.3) from frozen sperm in 1.5 mL TBS for 30 min at room temperature, under end-to-end agitation. The remaining solution was incubated with new immobilized sperm membrane proteins, allowing new interactions between SP proteins remaining in the supernatant and sperm membrane proteins. Ten rounds of interaction were performed until a consistent electrophoretic pattern of SP proteins was detected in the supernatant (Supplemental Fig. 1; online version only). Depletion was monitored by measuring the intensity of nine major bands and expressed relative to a band with less intensity changes than others (band #2, 10675.6 ± 1468.6 pixels per inch).

2.5. Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoresed in 15% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS-PAGE) at room temperature and 20 mA/gel in an SE250 device (Amersham GE, Buckinghamshire, UK), as described [18]. Samples were mixed with 5 × Laemmli sample buffer under non-reducing conditions. Molecular weight was estimated using protein low molecular weight standards (Benchmark, Invitrogen). Proteins were stained with Coomassie brilliant blue R-250 or silver nitrate [19], as indicated.

2.6. Protein concentration

Protein concentration of both SP and sperm fractions were determined by the Bradford method [20].

2.7. Identification of SP proteins capable of binding to sperm membrane molecules

Non-interacting and interacting SP proteins obtained by incubating SP (500 μg of protein) with immobilized sperm membrane proteins obtained from frozen-thawed semen, as described above (Section 2.4.), were washed with TBS, concentrated, and analyzed by SDS-PAGE. The interacting SP proteins were excised from a representative gel and identified by the MALDI-TOF ultraflex II (Bruker) procedure on the CEQUIBIEM pro-

teomic service (Buenos Aires, Argentina). Peptide mass fingerprinting was performed with the Mascot search engine (http://www.matrixscience.com, MatrixScience Ltd. UK) against the NCBI non-redundant protein database (http://ncbi.nlm.nih.gov) for the species *Ovis aries* and *Bos taurus*. Errors in peptide mass within 0.05% were allowed. Proteins that matched at least four peptides and had a Mascot score (based on MOWSE coefficient) > 71 were considered significant ($P < 0.05$). The amount of interacting SP proteins represented 10% of the proteins in whole SP, as estimated by protein concentration.

Specificity controls were performed by incubating immobilized sperm membrane proteins with either an excess of SP depleted of the interacting SP proteins, 500 μg ovalbumin, or 500 μg hemoglobin as non-related proteins, with or without the same amount of SP.

Identified proteins were analyzed using the following databases: InterPro Scan (www.ebi.ac.uk/Tools/InterProScan) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Expression data were obtained from UNIGENE, NCBI and other sources [21,22].

2.8. Binding of FITC-labeled interacting SP proteins to the sperm surface

Interacting SP proteins contained in the retained fraction were FITC-labeled using the FluoroTagTM FITC conjugation Kit (Sigma). Following dialysis against 0.1 M carbonate/bicarbonate buffer pH 9, proteins from the interacting fraction (0.5 mg) were labeled with 0.1 mg/mL FITC. Conjugated proteins were separated from the free dye by chromatography on Sephadex G-25, concentrated, and suspended in PBS containing 0.1% w/v BSA as carrier protein.

Thawed Percoll selected ram sperm were washed (0.1 × g, 10 min) and suspended in PBS at 25 °C. Sperm concentration was adjusted to 50×10^6 sperm/mL. Cells were incubated with 5 mg/mL BSA in PBS (blocking solution) for 15 min and washed twice by centrifugation. Then, the sperm suspension was incubated with: FITC-labeled proteins (1/10); 20% v/v SP; FITC-labeled proteins plus 20% v/v SP; or PBS. All incubations were done for 1 h at room temperature. After thoroughly washing with PBS, sperm were fixed with 2% paraformaldehyde, smeared, and mounted with PBS:glycerol (1:9). Specimens were examined with a Nikon fluorescent microscope equipped with a 100 X/1.25 oil E-plan objective.

2.9. Experimental design

2.9.1. Effect on sperm motility

An aliquot (160 μL) of sperm suspension (5×10^6 cells/mL) was incubated at 37 °C in 200 μL of final volume, with two groups of treatments.

2.9.1.1. Group A (effect of homologous and heterologous SP)

- Negative control: PBS (40 μL)
- Treatments:
  - 20% (v/v) SP from Frisian rams (homologous SP; 40 μL).
  - 20% (v/v) SP from Texel rams (40 μL).
  - 20% (v/v) SP from Corriedale rams (40 μL).

2.9.1.2. Group B (effect of SP fractions)

- Negative control: PBS plus 1.5 mg/mL fructose (40 μL).
- Treatments:
  - 20% (v/v) SP from Frisian rams (40 μL).
  - Interacting SP fraction corresponding to 40 μL SP from Frisian rams plus 1.5 mg/mL fructose in PBS (40 μL).
  - 20% (v/v) of SP depleted of interacting SP proteins (40 μL).

Note that when interacting SP proteins were assessed, fructose was added to the medium in a concentration equal to that present in SP from autumn, since SP also provided metabolic support to the sperm, particularly as an energy source [23]. Total motility and progressive motility ranged from 60 to 80% and from 50 to 70%, respectively, at 0 min of culture. At 0, 15, 30, and 45 min of culture at 37 °C, total sperm motility and progressive individual motility (%) were subjectively assessed (on a warm slide, at 37 °C), viewed with an optical microscope at × 400 magnification (Eclipse E200, Nikon, Japan). Sperm with progressive individual motility were those whose movement exhibited a defined linear course in the field of view. Approximately five fields per sample were examined by two independent viewers, and motility was estimated as a mean of the data. Since samples had varying motility at 0 min of culture, values of motility were presented as relative to 0 min time motility value [24].

2.9.2. Effect on sperm ultrastructure

An aliquot of the sperm suspension incubated with each treatment of Group B (100 μL) was fixed with 2.5% (v/v) glutaraldehyde in PBS for 4 h. Then, cells were centrifuged (700 × g, 10 min) and suspended in 200 μL of 1% osmium tetroxide (OsO_4) overnight.

Finally, they were dehydrated by incubating the pellet sequentially with 1 mL ethanol (70 to 100%) and acetone (100%) and embedded in Epon 812 (Pelco, USA). Ultrathin sections were obtained with an ultramicrotome apparatus (Ultracut, Reichert Jung), routinely stained by uranyl-lead citrate techniques, and 100 cells for each treatment were observed in a Zeiss 900 microscope at 80 Kv. The status of plasma membrane and mitochondria were specifically evaluated.

2.9.3. Statistical analyses

Sperm motility at each time was expressed as the proportion of motility at time 0. Data were assessed for normality distribution using the Shapiro-Wilk test (PROC UNIVARIATE) [25]. Group B data were subjected to logarithmic transformation to satisfy the assumption of normality. The PROC MIXED procedure was used for repeated analysis of covariance to test the effect of incubation time, treatment, and their interaction. Data are presented as least square means and the Tukey-Kramer test was used for comparison of means. The level of significance was set at $P < 0.05$.

3. Results

3.1. Effect of SP from various breeds on motility of cryopreserved sperm

Percoll®-selected, frozen-thawed sperm from Frisian rams were incubated with SP from Frisian, Corriedale, or Texel rams; total and progressive sperm motility were assessed at various times. There were no differences among relative total motility values of sperm incubated with SP of any breed, compared to sperm incubated with PBS (total motility relative to T0 at 15 min: Frisian SP 0.99 ± 0.11; Corriedale SP 0.90 ± 0.1; Texel SP 0.98 ± 0.3; PBS 0.82 ± 0.22; 30 min: Frisian SP 0.85 ± 0.28; Corriedale SP 0.81 ± 0.12; Texel SP 0.85 ± 0.29; PBS 0.68 ± 0.28; 45 min: Frisian SP 0.63 ± 0.44; Corriedale SP 0.58 ± 0.1; Texel 0.70 ± 0.20; PBS 0.50 ± 0.32). However, SP from the three breeds was able to maintain progressive motility ($P < 0.05$) at all incubation times (Fig. 1).

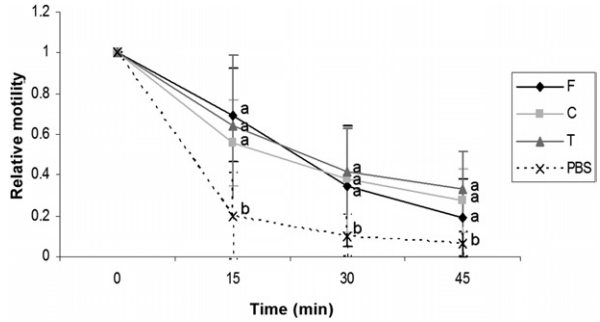

Fig. 1. Effect of SP (from various ram breeds) on sperm progressive motility. Motile sperm from Frisian rams were obtained by a Percoll gradient. Recovered sperm were incubated with 20% (v/v) SP from Frisian (F), Corriedale (C) or Texel (T) rams, or with buffer (PBS) as a control (n = 5). The percentage of progressive motile over total sperm was calculated at 0, 15, 30, and 45 min. Progressive motility was expressed relative to Time 0.

[a,b]Within a time, treatments without a common superscript differed ($P < 0.05$).

3.2. Identification of SP proteins that bound to the sperm membrane

To identify SP proteins that bound to sperm membrane molecules, sperm membrane proteins from a detergent extract were immobilized on a PVDF membrane and were used to retain SP proteins that were further analyzed by SDS-PAGE. Also, to investigate if these proteins were conserved among breeds, SP from Frisian, Corriedale or Texel rams was incubated with immobilized sperm membrane proteins of Frisian sperm. The membrane origin of the sperm extract was confirmed by Western blot using anti-cadherin. The sperm membrane extract had a unique band (115 kDa) that was absent in the cytosolic fraction (not shown). The membrane extract was composed mainly of proteins, but also cholesterol (0.21 μg/μL). Ponceau staining verified the presence of sperm membrane proteins on the PVDF membrane.

The electrophoretic patterns of SP proteins that interacted with the immobilized sperm membrane extract, as well as those that did not interact, were very similar among breeds (Fig. 2). Protein bands in the interacting fraction were excised from the gel and subjected to mass spectrometry analysis. Four relevant proteins were identified and their conserved domains were analyzed *in silico* (Table 1). To evaluate if binding of these proteins to the sperm membrane molecules was specific, a saturating amount of SP depleted from interacting SP proteins, or purified hemoglobin or ovalbumin, were used as potential competitors of binding in a competition experiment (Fig. 3). Changes in the electrophoretic pattern of the interacting proteins addressed the competition. Seminal plasma depleted of interacting SP proteins was obtained by repeated incubations of SP with immobilized sperm membrane proteins until no more proteins were retained, based on a comparison of major bands with consistent bands (Supplemental Fig. 1, on-line version

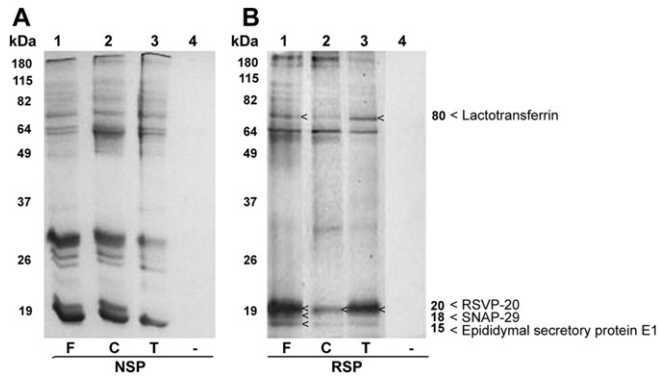

Fig. 2. Seminal plasma proteins capable of binding to sperm membrane molecules. Solubilized compounds of Frisian ram sperm membrane were immobilized on PVDF. These membranes were incubated either with SP from Frisian (F, lanes 1), Corriedale (C, lanes 2) or Texel (T, lanes 3) rams or with TBS (-, lanes 4) as a control. Membranes were washed to discard non-retained or non-interacting SP proteins (NSP) and the retained or interacting SP proteins (RSP) were eluted with 1.5 M NaCl. Non-interacting and interacting SP proteins were separated by SDS-PAGE and stained with Coomassie Blue (A) or Silver Nitrate (B). Proteins identified by MALDI-TOF are indicated with an arrowhead (n = 5).

only). As expected, neither the SP depleted of interacting SP proteins nor hemoglobin were capable of binding to the immobilized sperm membrane molecules, and consequently did not compete with the interacting SP proteins, manifested as an unaltered electrophoretic profile (Fig. 3A and C, lanes 4 and 5). In contrast, when ovalbumin, a cholesterol binding protein, was used as a competitor, binding of the interacting SP fraction was impaired (Fig. 3B, lane 5).

Immobilized sperm membrane proteins were also prepared from epididymal and fresh sperm. The SP proteins that interacted with immobilized sperm membrane proteins from epididymal sperm did not differ from those interacting with immobilized sperm membrane proteins from frozen sperm. However, no proteins were present in the interacting SP fraction when immobilized sperm membrane proteins from fresh sperm were assessed (Fig. 4).

To verify if the interacting SP proteins attached to the sperm surface membrane, and taking into account that the procedure used for membrane isolation cannot separate external from inner membranes, the retained protein fraction was FITC-labeled and incubated with intact sperm in the presence or absence of complete SP (Fig. 5). The labeled proteins bound to the sperm head and midpiece, with an intense fluorescence around the acrosome (Fig. 5A), that was displaced by SP (Fig. 5C).

Table 1
Analysis of the proteins identified by MS from the SP protein affinity fraction.

Name	Domains (InterPro Scan & BLAST)	Known functions	Origin
Epididymal secretory protein E1 [Bos taurus]	Lipid recognition domain: MD2 [20–149] Immunoglobulin-like beta-sandwich and related to fibronectin type III superfamilies [20–149]	Cholesterol binding protein [49]	Epididymis
Synaptosomal-associated protein 29 (SNAP-29) [Bos taurus]	tSNARE: family of membrane-associated proteins, membrane-bound probably by lipids [201–258]		Testis & prostate
Seminal vesicle protein precursor (RSVP-20) [Ovis aries]	Fibronectin type II: binding to cell surface and extracellular compounds, like collagen [65–150]. Kringle-like: membrane, proteins and phospholipid binding [61–151].	Binding to sperm surface by phosphatidylcoline and responsible for the protector effect over cryopreserved sperm [7]. Glycosylated protein involved in capacitation [7].	Seminal vesicle
Lactotransferrin [Ovis aries]	Peptidase S60-lactotransferrin: binding extracellular iron [25–695].	Binds to sperm surface [38]. Receptor on the sperm surface [39].	Testis & epididymis

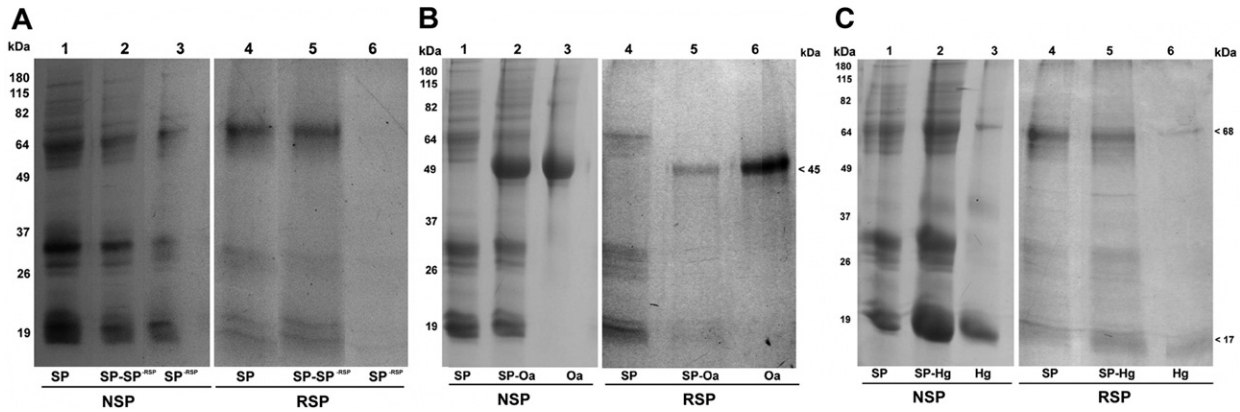

Fig. 3. Specificity binding assay: competition experiments. (A) PVDF membranes with immobilized sperm membrane proteins were incubated with SP (SP, lanes 1 and 4), one volume of SP + three volumes of SP depleted from retained or interacting SP proteins; (SP-SP^{-RSP}, lanes 2 and 5), or only SP depleted from retained or interacting SP proteins (SP^{-RSP}, lanes 3 and 6). (B) PVDF membranes with immobilized sperm membrane proteins were incubated with SP (SP, lanes 1 and 4), SP + ovalbumin (SP-Oa, lanes 2 and 5) or ovalbumin (Oa, lanes 3 and 6). (C) PVDF membranes with immobilized sperm membrane proteins were incubated with SP (SP, lanes 1 and 4), SP + hemoglobin (SP-Hg, lanes 2 and 5) or hemoglobin (Hg, lanes 3 and 6). In all competition experiments, membranes were washed and the retained or interacting SP proteins (RSP) were eluted with 1.5 M NaCl. The non-interacting and interacting SP proteins were separated by SDS-PAGE and stained with Coomassie Blue. Pictures are representative from three experiments.

3.3. Effect of bound proteins over sperm motility and ultrastructure

The ability of SP proteins that bind to the sperm membrane to repair the damage induced by freezing and thawing was evaluated by adding to thawed sperm an amount of interacting SP proteins comparable to the content of these proteins in whole SP and then, assessing sperm motility and ultrastructure (Fig. 6). Based on the fraction of progressive motile sperm obtained after 30 min of incubation, expressed as relative to time zero, it was clear that interacting SP proteins (with 1.5 μg/μL fructose) were as capable as whole SP of maintaining sperm progressive motility (0.59 $\pm$ 0.14 and 0.60 $\pm$ 0.09, respectively), and that motility was reduced ($P < 0.05$) when either SP depleted of interacting SP proteins or PBS were used (0.35 $\pm$ 0.05 and 0.16 $\pm$ 0.03, respectively).

Transmission electron microscopy showed that 50% of the cells incubated with interacting SP proteins depicted intact head membranes with well-condensed nuclei (Fig. 6C) and intact mitochondria (Fig. 6D); similar results were observed when sperm were incubated with whole SP (Figs. 6A and B). However, most of the sperm incubated with buffer had damaged membranes and ruptured acrosomes with dispersed acrosomal content (Fig. 6G) and mitochondria with reduced electron-density compared to controls, indicating damaged organelles and loss of content (Fig. 6H). Similar results were obtained when sperm were incubated with SP depleted of interacting SP proteins (Fig. 6E and F).

4. Discussion

In this study, we developed a method, using immobilized sperm membrane proteins, to study SP proteins that bound specifically to molecules of the sperm membrane. These proteins, which were designated interacting SP proteins, were as efficacious as whole SP to repair damaged sperm, protecting both sperm motility and ultrastructure. Moreover, some of these interacting SP proteins were conserved among breeds.

The beneficial effects of SP on cryopreserved ram sperm has been well documented. The effect of SP over ram sperm was studied by adding SP to fresh semen after cold shock (5 min at 10 °C) [7,8] and to frozen/thawed semen [9,11,14,26]. Moreover, the SP effect was also studied by adding SP before cold shock [2,27] or freezing [16,28,29], demonstrating that SP was able to minimize cryoinjury, by both protecting and repairing sperm damage at functional as well as ultrastructural levels [2,30].

Seminal plasma is composed of numerous molecules, as well as a vesicle fraction [31]. The abovementioned effects [12] were attributed to the protein fraction free from semen vesicles [32], since the pellet of membrane vesicles without proteins obtained by ultracentrifugation of SP lost its protective capacity

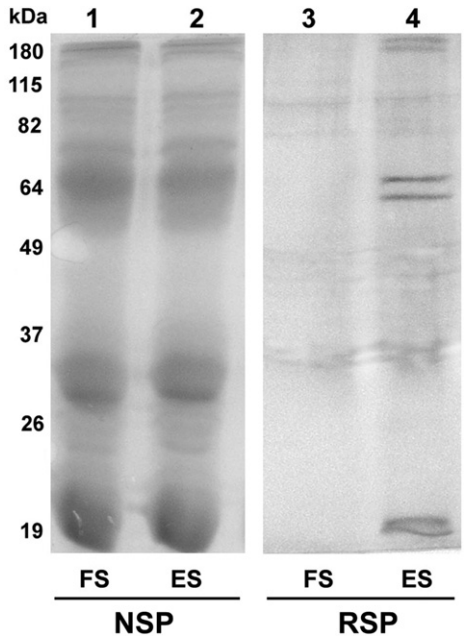

Fig. 4. Ability of ejaculated and epididymal sperm to bind SP proteins. Seminal plasma from Frisian rams was incubated with immobilized sperm membrane proteins from fresh ejaculated (FS, lanes 1 and 3) and epididymal sperm (ES, lanes 2 and 4). Non-interacting or non-retained SP proteins (NSP) and interacting or retained SP proteins (RSP) were obtained as in Fig. 2, separated by SDS-PAGE and stained with Coomassie Blue or silver nitrate, respectively.

[32]. Fractionation of ram SP proteins by exclusion chromatography yielded three fractions able to repair the effects of cold shock [8], but the most active fraction was the one containing the proteins RSVP14 and RSVP20. Supplementation of high-speed flowcytometer-sorted ram semen with increasing concentrations of a protein-rich fraction from SP, both before and after freezing, improved sperm parameters [16]. Recently, it was reported that supplementation of ram sperm with either crude SP or its protein component (> 10 kDa SPP) improved post-thaw viability [29], but did not consistently improve fertility after cervical insemination [33]. In the present study, a SP fraction of sperm surface-interacting proteins obtained with simple methodology, improved frozen/thawed sperm progressive motility and repaired damage to plasma membranes and mitochondria (similar to the effects of whole SP). However the SP fraction that lacked sperm surface-interacting proteins did not improve progressive motility, nor repair sperm membranes; therefore, SP was ineffective in the absence of interacting SP proteins The same effect has been reported for cold-shocked sperm incubated with whole SP [30].

The methodology (developed in this study) to isolate SP proteins capable of interacting with sperm membranes was a novel and simple procedure, which provides an interesting alternative. In the interacting SP fraction, three proteins previously reported to attach to the sperm surface were present: RSVP-20 [8], epididymal secretory protein E1 [34], and lactotransferrin [22]. The retained SP protein fraction containing these proteins was confirmed (fluorescence assay) to attach to the surface of intact sperm. Furthermore, this procedure facilitated development of a specificity-binding assay.

Both protective and repairing effects were reported to be highly specific, since neither SP proteins from bulls nor BSA restored ram sperm membrane integrity [35]. Similarly, not any SP protein was able to bind to the sperm membrane and the ones that bound could not be displaced by non-related proteins; therefore, the

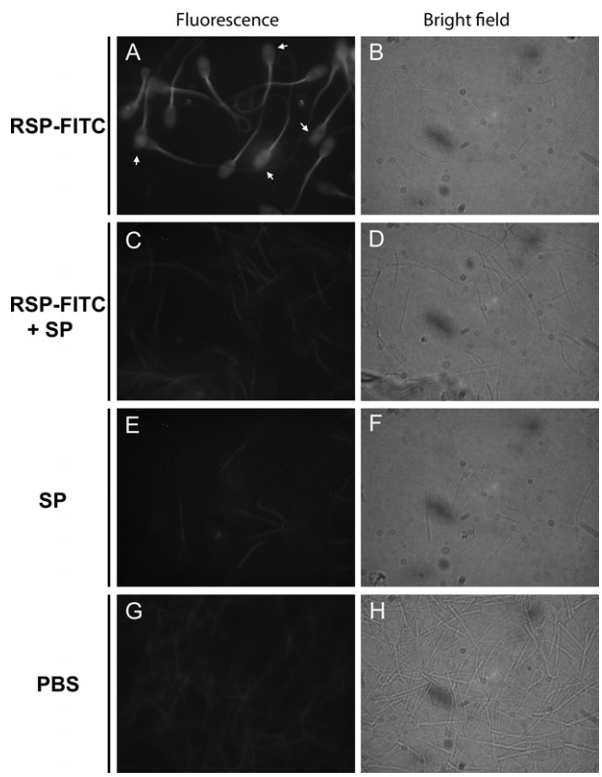

Fig. 5. Retained SP proteins specifically bind to sperm head. Proteins from the interacting fraction were FITC-labeled (RSP-FITC) using the FluoroTagTM FITC conjugation Kit (Sigma). Motile sperm were obtained by a Percoll gradient. Recovered sperm were blocked, washed and incubated either with RSP-FITC (RSP-FITC, panels A and B), RSP-FITC + 20% (v/v) SP (RSP-FITC +SP, panels C and D), 20% (v/v) SP (SP, panels E and F) or buffer (PBS, panels G and H). Cells were washed, fixed and observed with a Nikon fluorescent microscope (magnification: × 100). Left panels show fluorescence images (A, C, E and G) and right panels show their respective bright field (B, D, F and H). Arrows indicate fluorescent-labeled acrosomal membrane in A.

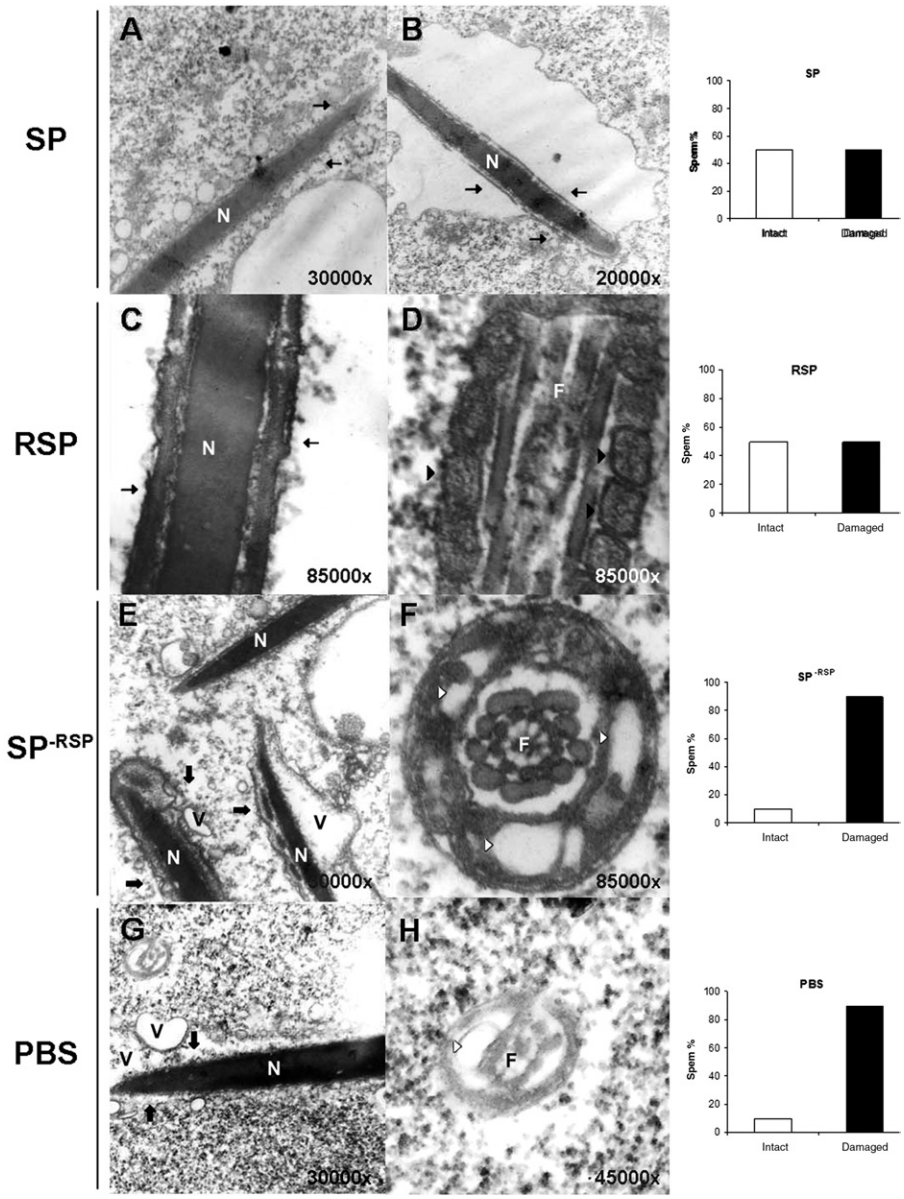

Fig. 6. Effect of SP fractions on sperm ultrastructure. Motile sperm were obtained by a Percoll gradient. Recovered sperm were incubated with 20% (v/v) SP from Frisian rams (SP, panels A and B), retained or interacting SP fraction (RSP, panels C and D), SP depleted of retained or interacting SP proteins (SP^{-RSP} panels E and F) and buffer (PBS, panels G and H) as a control. After 30 min, the sperm suspension was fixed and observed in a transmission electron microscope. The experiment was performed twice and 100 cells were observed for each treatment; A–H are representative pictures from each treatment. A, B, C, E and G are longitudinal sections of sperm heads; D is a longitudinal section of the mid-piece of sperm flagellum; and F and H are cross sections of the mid-piece of sperm flagellum. N, nucleus; F, flagellum; V, vesicle. Thin arrows indicate intact membranes, thick arrows indicate membrane vesiculation, filled arrowheads indicate intact mitochondria and empty arrowheads indicate damaged mitochondria. Magnification is indicated at the right bottom of each panel. Right panels are the percentage of intact and damaged cells for each treatment.

ability to identify SP proteins with these protective properties could be used to improve media for sperm cryopreservation and thawing. Four known sperm-binding proteins were identified in the interacting SP fraction: RSVP-20, which belongs to the family of bovine SP proteins (BSPs) [7]; Epididymal secretory protein E1, known to be involved in sperm epididymal maturation [34]; SNAP-29, reported to be expressed in

the testis and prostate (Unigene, NCBI, *Mus musculus* and *Homo sapiens*) [36]; and lactotransferrin, produced by Sertoli cells [22]. The latter attached to the sperm surface when the plasma membrane was functional [34,37,38] and, together with clusterin and semengolin, was associated with Eppin in a protein complex (EPC) [39]; this complex has been associated with regulation of capacitation [40,41]. Both RSVP-20 and SNAP-29 have fibronectin II domains, a motif included in bovine SP proteins (BSPs) [42].

Bovine SP proteins interacted with phospholipids in the sperm plasma membrane [43] and participated in destabilization (capacitation) and stabilization of the sperm membrane (decapacitation) [43–45]. These findings were consistent with the idea that the effect of SP was associated with the presence of coating components that maintained stability of the membrane up to the process of capacitation (decapacitation factors) [2,12,46,47]. It was noteworthy that addition of SP proteins prior to cold-shock not only improved sperm survival, but also decreased protein tyrosine phosphorylation, a hallmark of sperm capacitation [27]. That the same interacting SP fraction protein profile was observed for epididymal and frozen/thawed sperm, and that fresh sperm did not retain SP proteins, provided experimental evidence that SP proteins that bind to the sperm membrane might be decapacitating factors. This result can be easily explained if it is assumed that frozen/thawed sperm lose their coating components in a capacitation-like way, termed cryocapacitation, in which they become capable of adsorbing more SP proteins (when they are incubated with SP). Conversely, epididymal sperm have not been in contact with most SP coating components; therefore, they should allow SP proteins to bind to their surface. Instead, fresh ejaculated sperm were saturated with coating components. This was in agreement with our previous results that ram frozen/thawed sperm incubated with SP almost restored the protein electrophoretic profile of fresh sperm [14]. The effect of the interacting SP fraction was attributed to proteins that did not lose their bioactivity after affinity purification.

In addition, fructose in a concentration similar to that provided by SP was necessary to maintain sperm motility when only interacting SP proteins were added, but it was not enough for sperm incubated with buffer. This supported the idea that SP not only has protective proteins, but is also the metabolic support for sperm motility.

Since immobilized sperm membrane proteins were obtained by ultrasonication and detergent solubilization of sperm membranes, they might also include detergent-resistant-domains (also called rafts, [48]). As cholesterol was detected in this extract, we inferred that other lipids may also have been present. Moreover, based on the method used, perhaps these sperm extracts contained proteins other than plasma membrane proteins; however the SP proteins retained by them were indeed capable to bind to the sperm surface, as shown by the fluorescence assay, which was in consonance with the presence of RSVP20 in this fraction. Whether the proteins attached to specific receptors or not is still unclear, since the way by which SP proteins were bound to immobilized sperm membrane proteins has not been determined by our method. Interactions of SP proteins with lipids or proteins from the sperm membrane cannot be excluded.

That two of the proteins identified in the interacting SP fraction—RSVP20 and lactotransferrin—were conserved among three ram breeds was in consonance with the idea that these proteins contributed to maintaining sperm in a functional state. Since various breed combinations of SP and sperm were able to maintain sperm motility after cryopreservation, as the homologous SP did, was attributed to the presence of these proteins in all SPs tested.

Since SP proteins that bound to the sperm surface varied throughout the year, together with the ability of SP to prevent and protect cryodamage [14], the addition of interacting SP proteins from different ram breeds in their reproductive season after semen thawing was a promising tool to improve cryopreserved semen quality. Further work is underway in our laboratory to establish whether addition of this fraction to frozen/thawed semen increased pregnancy rates.

In conclusion, we demonstrated that SP proteins that bound to the sperm surface were conserved among ram breeds. Furthermore, when these proteins were added to frozen/thawed semen (along with an energy source), they repaired semen damage as well as whole SP, protecting both sperm motility and ultrastructure.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.theriogenology.2011.02.020.

Acknowledgments

This work was supported by grants from Universidad Nacional de Mar del Plata, ANPCyT PICTO-UNMdP 2004 (Project No. 8-375).

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