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Cryosurvival and In Vitro Fertilizing Capacity Postthaw Is Improved When Boar Spermatozoa Are Frozen in the Presence of Seminal Plasma From Good Freezer Boars

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ABSTRACT: The study evaluated the protective effect of seminal plasma (SP) added to freezing extender against cryopreservation injuries to boar spermatozoa. Pooled sperm-rich fractions collected from 9 fertile boars were frozen in 0.5-mL straws after being extended in a conventional freezing extender either alone or supplemented with 5% of SPs (SP1–SP4) collected from the sperm-rich fractions (diluted 1:1, vol/vol, in Beltsville Thawing Solution extender) from 4 boars (1–4) with known sperm cryosurvival (poor, moderate, and good sperm freezers). Cryopreservation injuries were assessed in terms of postthaw sperm motility (assessed by computer-assisted sperm analysis), viability (plasma membrane and acrosome integrity assessed simultaneously by flow cytometry), membrane lipid peroxidation (malondialdehyde [MDA] production), and the ability of thawed spermatozoa to fertilize in vitro–matured homologous oocytes. The addition of SP from good sperm freezers (SP3 and SP4) improved ($P < .01$) the motility and viability of thawed spermatozoa without any influence on MDA production. Moreover, SP from good sperm freezers also increased ($P < .05$) the percentage of penetrated (SP3) and polyspermic oocytes (SP4) with respect to the control. Neither the total amount of SP proteins, protein profiles, nor antioxidant capacity of the different SPs were related to the various cryosurvival/fertilizing capacities of the processed spermatozoa.

Key words: Cryopreservation, pig.
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It is well known that processing of spermatozoa such as with incubation, flow cytometric sorting, cooling, or cryopreservation affects sperm structures including membrane destabilization, which might ultimately result in cell death (reviewed by Maxwell and Johnson, 1999). In this respect, hardly 50% of the spermatozoa from an ejaculate survive the freezing and thawing process (reviewed by Roca et al, 2006b). Seminal plasma (SP) apparently minimizes these effects when a certain proportion of whole SP or particular SP components are added to the medium in which spermatozoa are suspended, including thawing media (Larsson and Einarsson, 1976; Vadnais et al, 2005a). Using this rationale, SP has proven beneficial to protect bull (Garner et al, 2001) and stallion (Aurich et al, 1996) spermatozoa during the cryopreservation process when added to the freezing extender. However, to the best of our knowledge, observations about the potential benefits of SP added to the freezing extender on post-thaw sperm cryosurvival have not been reported in pigs mostly owing to the customary removal of the bulk of SP before cooling in boar sperm cryopreservation protocols.

The SP composition varies among species as well as among males within the same species (reviewed by Strzezek et al, 2005). Thus, different SP profiles have been found between boars of different in vivo fertility (Flowers and Turner, 2001). Moreover, it has been suggested that the important variability among boars to sustain sperm cryopreservation could be related to differences in SP composition (Roca et al, 2006a), which may have a genetic origin (Thurston et al, 2002). In this way, as seen in other species, differences in SP protein profiles have been related to low and high sperm freezability in bulls (Jobim et al, 2004) and stallions (Zahn et al, 2005). Then, a logical hypothesis is that the supplementation of freezing extender with SP from boars with good sperm freezability could eventually

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improve the ability of boar spermatozoa to sustain freezing-thawing processes.

Therefore, the present study examined the possible protective effects of the addition of homologous SP to freezing extender on boar sperm cryosurvival. In addition, because the SPs were collected from ejaculates of boars with different sperm freezability, the amounts of the most relevant SP components were quantified to relate these to the encountered effect.

Materials and Methods

Reagents

Unless otherwise stated, all media components were purchased from Sigma Chemical Co (St Louis, Mo) and made up under sterile conditions in a laminar flow hood (HH48; Holten LaminAir, Allerod, Denmark) with purified water (18 MΩ - cm; Elgastat UHQPS; Elga Ltd, Lane End, Buckinghamshire, United Kingdom).

Boars and Ejaculate Collection and Processing

Procedures involving animals were in accordance with the recommendation of the Bioethics Committee of Murcia University. Healthy and fertile mature boars of various commercial hybrid lines undergoing regular semen collection for commercial artificial insemination and included in a sperm cryobank program were used. Boars were housed in individual pens in environmentally controlled buildings (commercial insemination station of PROINSERGA SA, Segovia, Spain). They were given ad libitum access to water and were fed commercial diets according to the nutritional requirements for adult boars.

Sperm-rich fractions were collected by the gloved-hand method, extended (1:1, vol/vol) in Beltsville Thawing Solution ([BTS] 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM $NaHCO_3$, and 3.35 mM EDTA), and evaluated by standard laboratory techniques (subjective analysis). Only ejaculates with at least 200×10^6 sperm per milliliter, at least 85% sperm with normal morphology, and at least 75% and at least 80% of motile and viable sperm, respectively, were selected for cryopreservation. The selected ejaculates were packed for shipping at 17°C and delivered 14–16 hours later to the Laboratory of Andrology of the Veterinary Teaching Hospital of the University of Murcia, Spain.

Collection and Storage of SP Samples

In a preliminary study in which 116 ejaculates from 29 mature fertile hybrid boars (4 ejaculates per boar) were cryopreserved, the boars were classified into 3 groups according to the post-thaw sperm survival assessed at 30 and 150 minutes after thawing as good, moderate, and poor freezers (Roca et al, 2006a). Four boars were selected among them as SP donors: SP1 was a poor sperm freezer (less than 40% of motile and viable spermatozoa), SP2 was a moderate sperm freezer (40%–60%), and SP3 and SP4 were good sperm freezers (more than 60%). SP was obtained during the cryopreservation process. After centrifugation ($2400 \times g$ for 3 minutes) of sperm-rich fractions diluted 1:1 (vol/vol) in BTS, the supernatant was collected and recentrifuged, filtered through a 10-μm nylon mesh filter to remove debris or clumped spermatozoa, and examined using phase microscopy to ensure no spermatozoa remained. Samples containing spermatozoa were refiltered. The supernatant (SP diluted in BTS) was stored frozen at −20°C until further use. Before using, SP was thawed at room temperature.

Sperm Cryopreservation

Ejaculates were cryopreserved using the straw freezing procedure described by Westendorf et al (1975) as modified by Thurston et al (2001) and Carvajal et al (2004). Briefly, pooled extended sperm-rich fractions were centrifuged at $2400 \times g$ for 3 minutes at 17°C, and sperm pellet was re-extended in lactose egg yolk (LEY) extender (80% [vol/vol] 310 mM β-lactose, 20% [vol/vol] egg yolk, 100 μg/mL kanamycin sulphate [pH 6.2], and 330 ± 5 mOsmol/kg) to yield to a concentration of 1.5×10^9 cells per milliliter. After further cooling to 5°C in 90–120 minutes, the spermatozoa were resuspended with LEY-Glycerol-Orvus ES Paste (LEYGO) extender (92.5% LEY + 1.5% Equex STM [Nova Chemical Sales Inc, Scituate, Mass], 6% glycerol [vol/vol] [pH 6.2], and 1145 ± 17 mOsmol/kg) to a final concentration of 1×10^9/mL. The resuspended and cooled spermatozoa were packed into 0.5-mL PVC-French straws (Minitüb, Tiefenbach, Germany) and frozen using a controlled-rate freezing instrument (IceCube 1810; Minitüb) as follows: cooled to −5°C at 6°C/min, from −5°C to −80°C at 40°C/min, held for 30 seconds at −80°C, cooled at 70°C/min to −150°C, and finally plunged into liquid nitrogen (LN_2). The straws remained in the LN_2 tank for at least 2 weeks before thawing, which was done in circulating water at 37°C for 20 seconds. Thawed spermatozoa from 2 straws per ejaculate were resuspended in BTS (1:2, vol/vol; 37°C) and incubated in a water bath at 37°C for 150 minutes.

Evaluation of Postthaw Sperm Quality

Measurements were done 30 minutes and 150 minutes after thawing. Postthaw spermatozoa were assessed for motility and viability (plasma membrane and acrosome integrity). Sperm motility was objectively evaluated using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer; Microptic, Barcelona, Spain) following the procedure described by Cremades et al (2005). Briefly, BTS-diluted thawed spermatozoa were further resuspended in BTS at a concentration of 30×10^6/mL. For each evaluation, a 4-μL sperm sample was placed in a prewarmed (39°C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), and 3 fields were analyzed to assess a minimum of 100 spermatozoa. The analysis yielded the following motility parameters: TSM (total motile spermatozoa, %), VCL (curvilinear velocity, μm/s), VSL (straight linear velocity, μm/s), VAP (average path velocity, μm/s); LIN (linearity, %), and ALH (amplitude of lateral head displacement, μm).

Plasma membrane and acrosome integrity was evaluated simultaneously using a triple fluorescent procedure described by Nagy et al (2003). Aliquots (100 μL each, about 3×10^6 cells) were transferred into culture tubes, and dyes were added at a final concentration of 25 nM SYBR-14 (100 μM stock solution in dimethyl sulfoxide [DMSO]; Component A of LIVE/DEAD Sperm Viability Kit, L-7011; Molecular Probes, Europe BV, Leiden, The Netherlands), 1 μg/mL of peanut agglutinin conjugated with phycoerythrin solution (1 mg/mL stock solution Phycoprobe R-PE-PNA, P44; Biomeda Corp, Foster City, Calif), and 12 μM propidium iodide (PI) (1.5 mM in phosphate buffer [PBS]; Component B of Sperm Viability Kit). Samples were mixed and incubated at room temperature (20°C–22°C) in the dark for 10 minutes. Just before analysis, 400-μL PBS was added to each sample and remixed before run through a flow cytometer. Flow cytometer analysis was performed by using a Coulter Epics XLTM (Coulter Corp, Miami, Fla). All dyes were excited by an argon ion 488-nm laser. SYBR-14 fluorescence (particles containing DNA, living cells) was detected with a 525 nm band-pass (BP) filter, PI signal (nonviable cells) was detected by a 620 nm BP filter, and PE-PNA fluorescence (cells with damaged acrosomes) was detected using a 575 BP filter. Acquisition and analysis were done using the EXPO 2000 (Coulter) software. Debris was gated out based on scatter properties and double gated out based on SYBR-14 or PI fluorescence. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Acquisition was stopped after recording 10 000 gated events, and event rates were kept around 800 cells per second. Only the percentage of live spermatozoa with intact acrosome was considered in results.

Measurement of Membrane Lipid Peroxidation

Membrane lipid peroxidation was estimated by the end-point generation of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) test following the procedure described by Carvajal et al (2004). Briefly, extended spermatozoa (250×10^6 cells in 1 mL) were mixed with 1 mL of cold 20% (wt/vol) trichloroacetic acid to precipitate proteins. The precipitate was pelleted by centrifugation ($1500 \times g$ for 10 minutes), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (wt/vol) TBA in a boiling water bath at 100°C for 10 minutes. After cooling, the absorbance was determined by spectrophotometry (UNICAM PU 8610 kinetics spectrophotometer; Philips, Eindhoven, Holland) at 534 nm. The results were expressed as a simple concentration of MDA (picomoles per 10^8 cells).

In Vitro Oocyte Maturation and Fertilization

The protocol used was that previously described by Gil et al (2004). Briefly, oocytes were obtained from ovaries of prepubertal gilts collected at a local slaughterhouse. The oocytes, surrounded by a compact cumulus mass and having evenly granulated cytoplasm, were matured (45–50 oocytes in 500 μL per well) in bovine serum albumin (BSA)-free NCSU (North Carolina State University)-23 medium supplemented with 10% (vol/vol) porcine follicular fluid, 0.1 mg/mL cysteine, and 10 ng/mL epidermal growth factor for 44 hours in 5% CO_2 in air at 39°C (22 hours with added hormones, 10 IU/mL equine chorionic gonadotropin [eCG] and 10 IU/mL human chorionic gonadotropin [hCG] [Intervet International BV, Boxmeer, The Netherlands], and then for another 22 hours without hormones). After the completion of in vitro maturation, batches of 30 denudated oocytes (0.1% hyaluronidase) were placed in 50-μL drops of preequilibrated in vitro fertilization (IVF) medium (modified tris-buffered medium [mTBM]; Abeydeera and Day, 1997). The dishes with the oocytes were kept in the incubator for about 30 minutes until spermatozoa were added for fertilization. A total of 100 μL of extended spermatozoa from 1 pool of 3 frozen-thawed (FT) straws was washed 3 times by centrifugation at $1900 \times g$ for 3 minutes in Dulbecco's Phosphate-Buffered Saline (DPBS) medium supplemented with 0.1% BSA, 75 μg/mL potassium penicillin G, and 50 μg/mL streptomycin sulfate (pH 7.2). At the end of the washing procedure, the sperm pellet was resuspended in IVF medium, and then 50 μL of this sperm suspension was added to the medium that contained oocytes so that each oocyte was exposed to 2000 spermatozoa. At 6 hours after insemination, oocytes were washed and transferred (30–35 oocytes per well) to a Nunc 4-well multidish containing 500 μL of NCSU-23 with 0.4% BSA and cultured at 39°C, 5% CO_2 in air for 10–12 hours. Then oocytes were mounted on slides, fixed in 25% (vol/vol) acetic acid in ethanol (24 hours at least), stained with 1% lacmoid in 45% (vol/vol) acetic acid, and examined under a phase contrast microscope at magnifications of 400×. The fertilization parameters evaluated were percentage of penetrated ([number of oocytes penetrated/total inseminated oocytes] × 100) and monospermic oocytes [(number of monospermic oocytes/total penetrated oocytes) × 100] and the number of spermatozoa per oocytes (mean number of spermatozoa in penetrated oocytes). Four replicates were done, and at least 120 oocytes per each FT sperm sample were evaluated.

Determination of SP Protein Profiles

Total protein concentration of SPs was estimated following the procedure described by Bradford (1976). SP proteins were isolated by reverse-phase high-performance liquid chromatography (RP-HPLC). Briefly, SP proteins were separated using an ETTAN LC HPLC system (Amersham Biosciences) and a Lichrospher RP100 C18 column (250 × 4 mm, 5-μm particle size; Merck, Darmstadt, Germany) eluted at 1 mL/min with a linear gradient of 0.1% trifluoroacetic acid in water (solution A) and acetonitrile (solution B), isocratically (5% solution B) for 5 minutes, followed by 5%–25% B for 10 minutes, 25%–60% B for 50 minutes, and 60%–70% B for 10 minutes). Protein detection was at 215 nm.

Assessment of the Antioxidant Components of SP

Total Nonenzymatic Antioxidant Capacity—Measurements were performed on deproteinized SP (adding 5% trichloroacetic acid on ice). Samples were subsequently centrifuged at $10\,000 \times g$, the pellet was discarded, and measurements were performed on the supernatant. Total nonenzymatic antioxidant capacity (TAC) was measured using the ABTS (2,2′-Azino-di-(3-ethylbenzthiazoline sulphonate)/horseradish per-

oxidase [HRP] decoloration method (Cano et al, 1998). This method is based on the capacity of different components to scavenge the ABTS radical cations ($ABTS^{\cdot+}$) compared with a standard antioxidant (Trolox) in a dose-response curve. Preformed $ABTS^{\cdot+}$ was obtained by mixing 1 mmol/L ATBS, 60 μmol/L H_2O_2, and 0.25 μmol/L HRP in 50 mM sodium phosphate buffer (pH 7.5). Then, 10 μL of SP was added to the reaction medium (200 μL) and the decrease in absorbance, directly proportional to the $ABTS^{\cdot+}$ quenched, was determined at 730 nm at 5 and 30 minutes in a 96-well microplate reader (Anthos 2010, Rosys Anthos, Germany) Data were expressed as micromoles of Trolox equivalents.

Enzymatic Antioxidant Activity—For estimation of the enzymatic antioxidant activity, SP samples (1 mL) were centrifuged at 10 000 × *g* for 5 minutes. Then the supernatant was separated and kept in ice until the analysis. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) activities were measured as described by Martí et al (2003) for ram spermatozoa. The absorbance changes were monitored with an UV-Vis Hitachi spectrophotometer (U-2000; Hitachi Ltd, Tokyo, Japan) measuring the NADPH oxidation or (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) XTT sodium salt reduction at 340 or 470 nm, respectively. One enzyme unit (IU) was defined for GPx and GR as the oxidation of 1.0 μmol/min of NADPH at 25°C at pH 7.5 or as the amount of SOD capable of transforming 1.0 μmol/min of $O_2^{\cdot-}$. The enzyme activity was expressed in units per milliliter per milligram (U/mL/mg) of protein.

Experimental Design

Extended sperm-rich fractions (1:1, vol/vol in BTS) from 9 boars were pooled and centrifuged. After centrifugation, the pooled sperm pellet was split into 5 aliquots and then extended with LEY alone (control) or LEY supplemented with 5% (vol/vol) of SP from poor (SP1), moderate, (SP2), and good sperm freezers (SP3–SP4). Eight replicates were done. The SP inclusion level was chosen based on its demonstrated efficacy in a preliminary experiment where other concentrations were also tested (unpublished data).

Statistical Analysis

Statistical analyses were performed by SPSS version 14 (SPSS Inc, Chicago, Ill). Data were analyzed as a split plot design using a mixed-model analysis of variance (ANOVA). To fulfill the assumption of a normal distribution, percentage data of postthaw sperm quality were log-transformed before statistical analysis. The effect of SP on the postthaw sperm survival was examined according to a statistical model that includes the fixed effects of postthaw evaluation time (30 and 150 minutes), SP donor (0 to 4), and the random effect of replicate. There were no interactions between evaluation time and SP donor. Therefore, data of the postthaw evaluation times were combined and analyzed as a complete data set. The antioxidant composition of SP was analyzed by 1-way ANOVA. Data of IVF were analyzed according to statistical model, including the fixed effect of SP donor and the random effect of replicate. Data in percentages of penetrated and monospermic oocytes were modeled according to the binomial model of parameters before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni or Turkey's post-hoc test and were considered to be significant when the *P* value was less than .05. Results are presented as mean ± SEM.

Results

Effects of the addition to the freezing extender of SP from poor, moderate, or good sperm freezers on the motility and viability of postthawed spermatozoa are summarized in Table 1. Irrespective of the source, the addition of SP to the freezing extender increased the percentage of motile sperm in all samples, being the improvement statistically significant ($P < .01$) in samples frozen in the presence of SP from good sperm freezers (SP3 and SP4). The same trend was observed for postthaw sperm viability. The percentage of live sperm with intact acrosome (VIA) was also significantly ($P < .01$) higher in sperm samples frozen in the presence of SP from good sperm freezers (SP3 and SP4). The presence of SP in the freezing extender did not modify ($P > .05$) the kinematic pattern of postthaw motile spermatozoa.

The addition of SP on the freezing extender had no effect ($P > .05$) on MDA production of FT spermatozoa (Figure 1). Furthermore, the maximum and minimum levels of MDA were observed in sperm samples frozen with SP from good freezers (119.4 and 86.0 pmol MDA per 10^8 for SP3 and SP4, respectively).

The addition of SP to the freezing extender influenced ($P < .01$) the IVF parameters evaluated (Figure 2). Sperm samples frozen with SP3 (good freezer) showed the highest penetration rate. Moreover, SP4 (good freezer) also increased the number of spermatozoa per oocyte ($P < .01$) compared with control and consequently decreased ($P < .01$) the percentages of monospermic oocytes. In contrast, no significant ($P > .05$) differences on IVF parameters were found between sperm samples frozen in the presence of SP from moderate or poor freezers and control (sperm samples frozen without SP).

The activities levels of SOD, GPx, GR, and TAC in the 4 SP sources are shown in Table 2. The enzymatic activity levels of SOD, GPx, and TAC were highly variable ($P < .01$) among SPs, without any definite pattern related to sperm freezability of SP donors. Moreover, GR concentration was relatively inappreciable in all SPs, without a significant difference ($P > .05$) among them.

The total protein content varied greatly irrespective of the SP origin (Table 3). Among individual proteins, PSP-I and PSP-II were, in this order, the more abundant

Table 1. *Postthaw sperm motility and viability of frozen-thawed boar sperm samples processed in the presence of exogenous seminal plasma (5% vol/vol; SP1–SP4) collected from 4 boars with different sperm freezability**

Sperm Parameters†	Source of Seminal Plasma					SEM	Probability
	Control	SP1	SP2	SP3	SP4		
TSM, %	50.3a‡	54.4ab	54.9ab	56.7b	59.5b	2.5	.001
VCL, μm/s	64.6	61.8	63.1	66.1	64.8	2.4	NS§
VSL, μm/s	43.7	40.6	41.1	44.8	43.2	2.0	NS
VAP, μm/s	50.1	47.5	48.1	51.3	50.2	2.2	NS
LIN, %	66.6	65.4	65.8	66.7	66.7	1.4	NS
ALH, μm	2.0	2.0	2.1	2.1	2.1	0.7	NS
VIA, %	49.5a	52.3ab	52.4ab	57.2bc	60.0c	3.4	.001

* Values are mean ± SEM of 2 separate measurements at 30 and 150 minutes after thawing in sperm samples incubated during 150 minutes in a water bath at 37°C. The sperm sample indicated as control was frozen in extender without additional seminal plasma. Data are from 8 replicates.

† Motility characteristics obtained from computer-assisted sperm analysis: TSM indicates total motile spermatozoa; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement; and VIA, live sperm with intact acrosome assessed by a fluorescent triple stain.

‡ Letters a–c denote a significant difference within treatments ($P < .05$).

§ NS indicates nonsignificant.

proteins in the 4 SPs evaluated. The reverse-phase separated PSP proteins followed the same pattern as the total protein profile, showing the highest (PSP-I) and lowest (PSP-II) quantity of these proteins in the SPs from the boars considered good sperm freezers (Figure 3).

Discussion

Removing the bulk SP by centrifugation is a necessary step in boar sperm cryopreservation protocols to concentrate the sperm population so that it can be resuspended in adequate proportions of freezing extender (Carvajal et al, 2004). Therefore, boar spermatozoa are exposed to bulk and diluted SP until centrifugation and thereafter deprived from large quantities of the SP. Because exposure to autologous SP before (Tamuli and Watson, 1994) or to homologous SP after processing (eg, postthaw; Larsson and Einarsson, 1976) has beneficial effects on viability, it is of utmost importance to know if such benefits are also issued during cooling and freezing, a matter largely unknown thus far. In others species, such as bovine (Garner et al, 2001), caprine (Azerêdo et al, 2001), equine (Katila et al, 2002; Aurich et al, 1996), or red deer (Martinez-Pastor et al, 2006), SP has improved sperm cryosurvival. Although

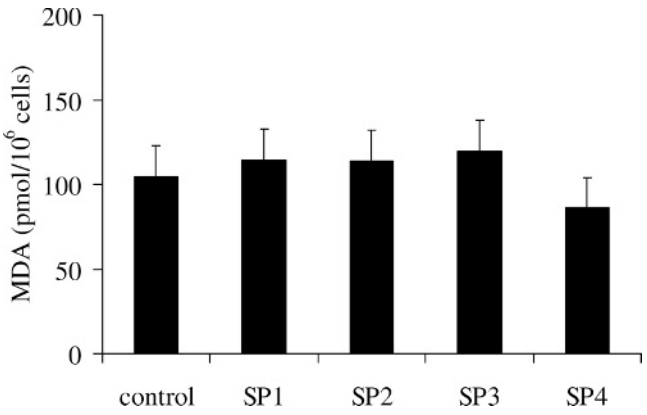

Figure 1. Concentration of malondialdehyde (MDA) in frozen-thawed boar sperm samples processed in the presence of exogenous seminal plasma (SP) (5% vol/vol; SP1–SP4) collected from 4 boars with different sperm freezability. Values are mean ± SEM of 2 separate measurements done at 30 and 150 minutes after thawing in sperm samples incubated during 150 minutes in a water bath at 37°C. The sperm sample indicated as control was frozen in extender without additional SP.

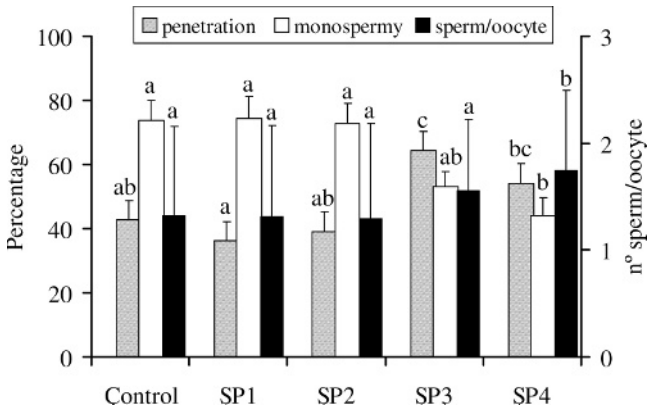

Figure 2. Parameters during in vitro homologous oocyte penetration testing of frozen-thawed boar sperm samples processed in the presence of exogenous seminal plasma (SP) (5% vol/vol; SP1–SP4) collected from 4 boars with different sperm freezability. The sperm sample indicated as control was frozen in extender without additional SP. The parameters evaluated include penetration and monospermic rates and the mean number of spermatozoa per oocyte (n° sperm/oocyte). Data are means ± SEM of 4 replicates. Letters a–c denote a significant difference within treatment. ($P < .05$)

Table 2. *Mean ± SEM enzymatic (superoxide dismutase [SOD], glutathione peroxidase [GPx], and glutathione reductase [GR]) and nonenzymatic (TAC) antioxidant activity levels of the seminal plasma (SP1–SP4) collected from 4 boars with different sperm freezability (poor to good) and used in the present study as exogenous supplement during freezing of boar spermatozoa*

Seminal Plasma Sources*	SOD, U × 10^{-3}/mL/mg protein	GPx, U × 10^{-3}/mL/mg protein	GR, U × 10^{-3}/mL/mg protein	TAC, μmol/L of Trolox Equivalents
SP1	54.9 ± 1.3a†	10.3 ± 8.5a	0.5 ± 0.2a	726.66 ± 12.13a
SP2	37.5 ± 0.3b	13.7 ± 7.5b	0.5 ± 0.1a	866.00 ± 25.32b
SP3	25.8 ± 1.6c	6.9 ± 3.7c	0.1 ± 0.2a	640.33 ± 30.07a
SP4	96.9 ± 0.4d	14.3 ± 6.0b	0.6 ± 0.0a	636.00 ± 27.78a

* SP1 was collected from a boar poor sperm freezer, SP2 from moderate, and SP3 and SP4 from good sperm freezers.
† Letters a–d denote significant differences among seminal plasma sources ($P < .05$).

SP has never been used as a freezing extender additive in pigs, the possible role in sperm freezability seems controversial. In fact, Kawano et al (2003) showed an improvement in boar sperm cryosurvival when SP was removed immediately after collection (within 20 minutes) whereas Pursel et al (1973) and Tamuli and Watson (1994) showed that the incubation of spermatozoa in its own SP at room temperature during a prefreezing holding time allowed spermatozoa to resist better eventual cold shock. Differences among boars in the composition of SP could explain the above disparity between results. In this respect, the present study shows that the effect of SP added in the freezing extender on sperm cryosurvival is SP-donor dependent. Hence, the percentages of motile and viable FT spermatozoa were only significantly higher in the sperm samples frozen in the presence of SP from good sperm freezers. The different effectiveness of these SPs, clearly donor-related, has already been reported. Caballero et al (2004) showed that SP can exert a positive or negative effect on highly diluted boar spermatozoa depending on the boar. In the same way, Henault and Killian (1996) showed different in vitro penetration abilities of SP-incubated bull spermatozoa according to SP donors. Moreover, Aurich et al (1996) achieved similar results to ours, showing that the addition of SP from good sperm freezers to freezing extender improved the post-thaw sperm survival in stallions.

It is clear that the composition of SP varies among species, among males within the same species, and among ejaculates within the male (reviewed by Strzezek et al, 2005). Thus, an adequate knowledge of the composition of SPs used in the present study should help to understand the different response of spermatozoa to cryopreservation. Proteins are among the major components of SP, and they affect sperm functionality. For instance, Barrios et al (2005) have recently shown that SP proteins are capable of protecting ram spermatozoa against cold shock. On the other hand, heparin-binding SP proteins (HBPs) have shown a dose-dependent adverse effect on buffalo epididymal spermatozoa during cryopreservation (Harshan et al, 2006). In the boar, spermadhesins represent about 75% of the total protein content of SP, with PSP-I and PSP-II being the most abundant proteins, amounting to up to 50% of the total proteins (Calvete et al, 1995). We have reported that these proteins can preserve sperm quality of highly diluted fresh and FT spermatozoa (Centurión et al, 2003; Caballero et al, 2004; García et al, 2006). However, although the SP proteinogram differs among SPs, those differences seemed unrelated to the different protective effects observed. Moreover, this approach had been assessed in a previous experiment in which we showed that the supplementation of the freezing extender with PSP-I/PSP-II or HBP did not affect postthaw sperm survival positively or negatively (Cremades et al, 2004). Confounding results were also found by Peña et al (2006) in relation to portions of the ejaculate with clear differences in SP contents. The lack of any effect should be due to either the presence of

Table 3. *Total protein content and contents of individual proteins (mg/mL) in the seminal plasma of individual boars (n = 4; SP1–SP4) with different sperm freezability; each seminal plasma was used in the present study as exogenous supplement during freezing of boar spermatozoa*

Seminal Plasma Sources	Seminal Plasma Proteins							
	Total Protein	AQN1	AQN3	PSP-I	PSP-II	AWN1	AWN2	Others
SP1	11.0	0.5	0.3	5.2	1.6	0.2	0.7	2.4
SP2	9.5	0	0.1	4.2	2.8	0.2	0.4	1.8
SP3	14.2	0.3	0.4	6.2	3.8	0.1	0.7	2.6
SP4	5.1	0.1	0.1	2.1	1.5	0.2	0.2	0.8

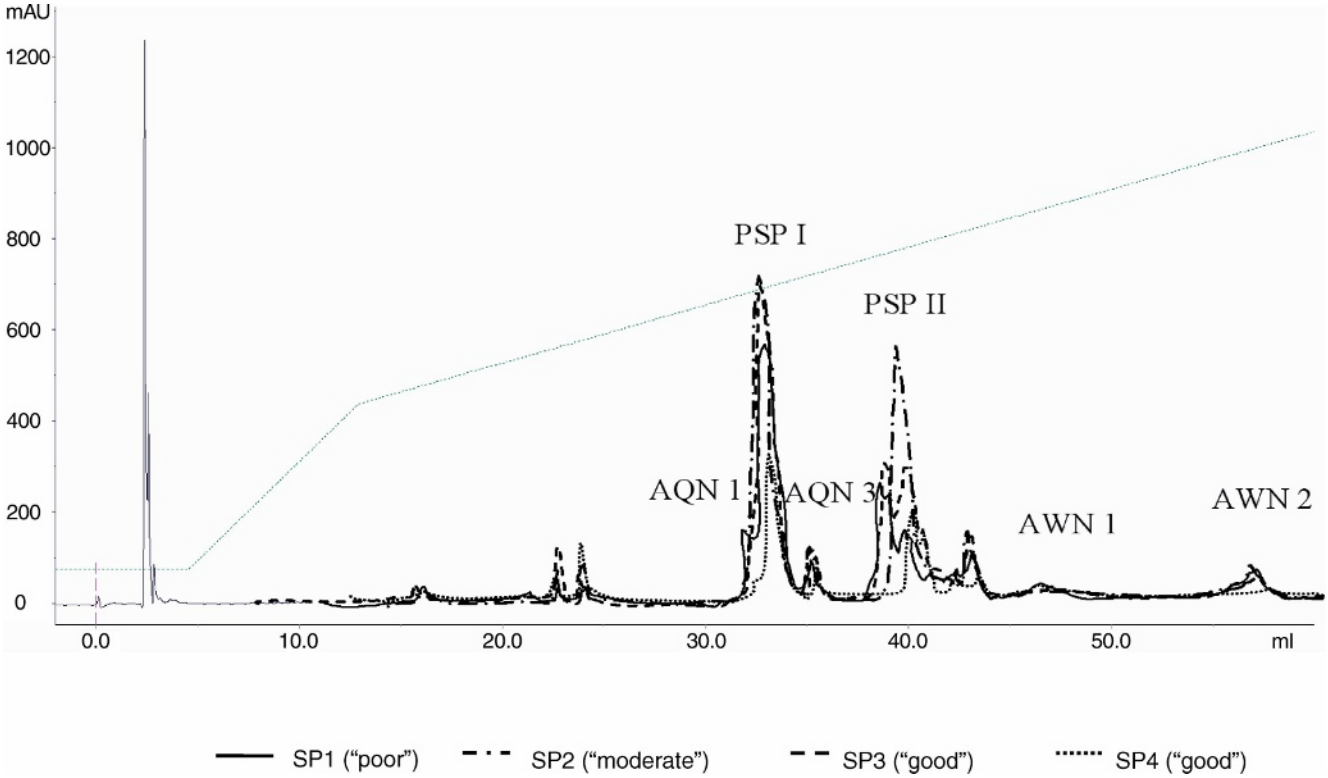

Figure 3. Reverse-phase high-performance liquid chromatography protein profiles of the seminal plasma (SP1–SP4) from the 4 boars exhibiting distinct sperm freezability (poor to good).

other substances in SP or in the freezing extender, which limit or mask the action of those proteins, avoiding its adsorption onto the sperm membranes or suppressing its activity (Vadnais et al, 2005a). In fact, it has been shown that the activity of bovine SP proteins on sperm membranes is inhibited by the formation of stable complexes with egg yolk lipoproteins (Manjunath et al, 2002; Bergeron and Manjunath, 2006). In this respect, it should be taken into account that freezing extenders used for cryopreserving boar spermatozoa contain 20% egg yolk.

Besides the above-mentioned effects of certain proteins, boar SP contains a wide variety of organic and inorganic components capable of modulating different sperm functions, including protection from the negative effects of reactive oxygen species (Strzezek, 1999; Strzezek et al, 2005). Lipid peroxidation, originated by raeactive oxygen species, has been claimed as one of the main causes of sperm damage during freezing and thawing processes (Bilodeau et al, 2000). In addition, supplementation of freezing extenders with antioxidant components has been demonstrated to be effective for the improvement of postthaw boar sperm quality (ie, vitamin E [Peña et al, 2003] and catalase, SOD, and butylated hydroxytoluene [Roca et al, 2004; 2005]). Thus, qualitative and/or quantitative differences in SP components with antioxidant properties could explain the distinct effectiveness of different SPs. To test this possibility, we have determined the concentrations of the more relevant antioxidants components present in boar SP (Strzezek et al, 2005). As expected, the concentration of the different antioxidants showed significant differences among the 4 boars providing SP, although always within the normal range of boar SP according to the values previously showed by Strzezek (2002). However, it is noteworthy that the antioxidant properties of the SPs sources were not related to postthaw sperm quality. Moreover, no differences in lipid peroxidation, indirectly measured as MDA production, were found among sperm samples. Therefore, we conclude that the antioxidant property of SPs was not a major factor responsible for the distinct influence of SP on postthaw sperm survival.

Among the major protective effects attributed to SP are preventing spermatozoa from undergoing capacitation and to reverse capacitation in a sperm population that has apparently already undergone this process (Vadnais et al, 2005a,b; Kaneto et al, 2002). Fresh or FT boar spermatozoa, incubated with whole SP or with specific SP proteins, have a limited ability to penetrate oocytes under in vitro conditions (Suzuki et al, 2002; Caballero et al, 2004). In the present study, although

without a postthaw sperm quality-related pattern, SP added to the freezing extender did not decrease the ability of thawed spermatozoa to penetrate oocytes under in vitro conditions. Moreover, the highest rates of penetrated oocytes were obtained from sperm samples frozen in the presence of SP. This observation could be related to the contact time of SP with the spermatozoa. In the present experiments, SP was added to the freezing extender and FT spermatozoa were thoroughly washed before IVF. Thus, the IVF results achieved seem to be related primarily to the higher proportion of spermatozoa surviving the cryopreservation process, in the presence of some sources of SP, rather than to the possible role of SP on the sperm capacitation process. Further studies are needed to clarify any other eventual relation.

In conclusion, the addition of SP to the freezing extender can improve boar sperm cryosurvival. However, the beneficial effect depends on SP source, being only significant when SP was collected from boar with good sperm freezability. Looking at the SP composition, it is likely that neither protein profiles nor the antioxidant capacity of SP can explain per se the differences on effectiveness among SPs. Therefore, further investigations are necessary to elucidate which components of SP and mechanisms linked to the SP are responsible for the potential protective effect on boar sperm freezability.

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