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Does Seminal Plasma PSP-I/PSP-II Spermadhesin Modulate the Ability of Boar Spermatozoa to Penetrate Homologous Oocytes In Vitro?

IGNACIO CABALLERO,* JUAN M. VAZQUEZ,* MARIA A. GIL,* JUAN J. CALVETE,† JORDI ROCA,* LIBIA SANZ,† INMACULADA PARRILLA,* EVA M. GARCIA,* HERIBERTO RODRIGUEZ-MARTINEZ,*‡ AND EMILIO A. MARTINEZ*

*From the *Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain; †Institute of Biomedicine, C.S.I.C., Valencia, Spain; and ‡Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.*

ABSTRACT: Low concentration (0.15 mg per million of spermatozoa) of seminal plasma–derived PSP-I/PSP-II spermadhesin heterodimer is able to preserve the viability of highly extended boar spermatozoa. Whether spermatozoa also keep their fertilizing capacity is not yet known. The present study evaluated the effect of exposing freshly extended and frozen-thawed boar spermatozoa (10 million/mL) to PSP-I/PSP-II (1.5 mg/mL) for 30 or 120 minutes on sperm characteristics and the outcome of in vitro penetration of immature (IM) and in vitro matured (IVM) homologous oocytes, aiming to identify this spermadhesin as a suitable modulator for sperm-handling protocols. Although exposure to the heterodimer improved sperm viability and motility without increasing the levels of sperm acrosome exocytosis in both freshly extended and frozen-thawed spermatozoa, this pretreatment did not affect sperm penetration rates or sperm numbers per oocyte when pretreated fresh spermatozoa were coincubated with IM or IVM oocytes compared with controls. When cryopreserved spermatozoa were tested, however, on IVM oocytes, already a 30-minute preincubation exposure to PSP-I/PSP-II showed a significant blocking effect on penetration rate (from 90% to 32%, $P < .05$) and on mean sperm numbers per oocyte (2.9 to 1.6, $P < .05$). To disclose the nature of this paradox, frozen-thawed spermatozoa were cleansed (by centrifugation in saline bovine serum albumin or through Percoll density gradient separation) and the procedure repeated. Oocyte penetration (but not number of spermatozoa per oocyte) increased ($P < .05$) when spermatozoa were cleansed with Percoll compared with either washed or unwashed controls (53% vs 13% vs 31%, respectively). In addition, the percentages of polyspermic oocytes remained lower than control (38.5% vs 68.7%, respectively; $P < .05$). In conclusion, the results confirm that exposure of fresh or frozen-thawed boar spermatozoa to a low dose of seminal PSP-I/PSP-II spermadhesin preserves sperm viability and motility in vitro. Although there was no obvious influence of the heterodimer on the capability of freshly extended boar spermatozoa to penetrate homologous oocytes (either IM or IVM), PSP-I/PSP-II exerted a deleterious effect when frozen-thawed spermatozoa were used to penetrate IVM oocytes. Such an effect of cryopreservation seems to a certain extent reversible, since cleansing of the sperm surface decreased, at least partially, this blocking effect, increasing both penetration and the monospermic rates.

Key words: Seminal plasma, preservation, sperm viability, IVF, pig.

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Seminal plasma (SP), the fluid in which mammalian spermatozoa are suspended in semen, is a complex mixture of secretions that originate from the testes, epididymides, and male accessory sexual glands. The SP contains factors that influence both spermatozoa and the female genital tract during sperm transport (Shivaji et al, 1990; Yanagimachi, 1994; Waberski et al, 1995). In particular, SP proteins play a role in the modulation of sperm function before they reach the oocyte(s) at the tubal site of fertilization, during gamete recognition, and when spermatozoa and oocytes bind at fertilization (Calvete et al, 1995a). In boars, the major protein component of the SP is the spermadhesin family (Calvete et al, 1995a; Töpfer-Petersen et al, 1998), a group of (glyco)proteins built by a single CUB domain architecture (Romero et al, 1997), coating the sperm surface (Dostálová et al, 1994). According to their binding properties, spermadhesins can be divided into 2 groups, depending on their ability to either bind heparin (AQN-1, AQN-3, AWN) or not (PSP-I/PSP-II heterodimer). The PSP-I/PSP-II heterodimer appears to preserve in vitro membrane integrity, motility, and mitochondrial activity of highly extended spermatozoa for as long as these are exposed to the heterodimer

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Correspondence to: Dr Vazquez, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain (e-mail: vazquez@um.es).

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(Centurión et al, 2003). Spermadhesin PSP-I/PSP-II has a similar protective effect as that reported for boar spermatozoa on the addition of either bulk SP from selected males (Maxwell et al, 1997; Maxwell and Johnson, 1999) or SP components of low molecular weight (Ashworth et al, 1994). The use of an isolated heterodimer, the PSP-I/PSP-II, has the advantage of avoiding the inherent variability shown by bulk SP among males or ejaculates from one and the same male (Killian et al, 1993; Ashworth et al, 1994; Rozeboom et al, 2000).

Porcine in vitro fertilization (IVF) is still considered suboptimal compared with the outcome of this reproductive biotechnology on other species, with unacceptable levels of polyspermia associated with high penetration rates. Although ejaculated boar spermatozoa, surrounded by SP, are able to produce high penetration levels when coincubated with homologous oocytes (Martinez et al, 1996), the level of fertilization is lower with ejaculated than with epididymal spermatozoa (Rath and Niemman, 1997), indicating that use of bulk SP on IVF is not beneficial (Bonilla et al, 1996; Rath and Niemman, 1997; Maxwell et al, 1998). Coincubation of immature (IM) or in vitro matured (IVM) oocytes with 1.5 mg/mL of PSP-I/PSP-II in the IVF medium significantly decreased penetration rates by untreated boar spermatozoa (Caballero et al, unpublished data). These data, together with the fact that the PSP-II subunit of the PSP-I/PSP-II heterodimer exhibits binding affinity for zona pellucida (ZP) receptors (Calvete et al, 1995b), suggest that the heterodimer can block penetration, at least in vitro. However, the PSP-I/PSP-II spermadhesin binds loosely to the sperm surface (Calvete et al, 1995b), and, consequently, an easy removal should be expected in vitro and during sperm coincubation with the oocytes. Therefore, preincubation of spermatozoa with PSP-I/PSP-II should not affect the interaction between spermatozoa and the oocyte. These properties, along with the sperm protective effect, point to PSP-I/PSP-II as a potential candidate for pretreatment of manipulated spermatozoa (extended, stored, or deep frozen) aimed at promoting sperm survival and performance in vitro. This is especially important when technologies such as sex selection of spermatozoa by flow cytometric sorting or sperm cryopreservation are used, procedures known to induce deleterious changes in boar spermatozoa.

Owing to such a potential beneficial effect on sperm viability and since the effect of sperm pretreatment with the PSP-I/PSP-II heterodimer on IVF has not been investigated, the present study examined the in vitro penetration ability (penetration rate and number of spermatozoa per oocyte) of fresh and frozen-thawed boar spermatozoa (10×10^6/mL) on IM and IVM homologous oocytes following preincubation with low doses of the heterodimer (1.5 mg/mL) for 30 or 120 minutes. In addition, the rate of polyspermia was determined when IVM oocytes were penetrated.

Methods[1]

Isolation of Spermadhesin PSP-I/PSP-II Heterodimer From Boar SP

All experiments were performed with the SP from 4 sexually mature boars, which had previously sired offspring. Sperm-rich ejaculate fractions were obtained using the gloved-hand method and SP was separated from spermatozoa by centrifugation at $3800 \times g$ for 15 minutes at 20°C. The supernatants were sequentially filtered through 10- and 1.2-μm filters and pooled.

The PSP-I/PSP-II heterodimer was isolated from the non–heparin-binding fraction of SP by size exclusion chromatography on a 2000 × 5-cm Sephadex G-50 column equilibrated in 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, and 0.025% sodium azide (pH 7.4) (Calvete et al, 1995b). The identity and purity of the protein preparation were assessed by *N*-terminal sequence analysis (using a 472 automated protein sequencer, Applied Biosystems, Langen, Germany) and MALDI-TOF mass spectrometry using a Voyager DE-Pro mass spectrometer (Applied Biosystems). A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Protein concentration was determined spectrophotometrically using a molar absorption coefficient (27 332 M^{-1} cm^{-1}) determined by Menendez et al. (1995) or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 hours at 106°C in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyzer (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilized, and stored at −20°C until used.

Handling, Incubation With the Heterodimer PSP-I/PSP-II, and Evaluation of Spermatozoa

Fresh semen was received in commercial doses for artificial insemination (collected from different boars of proven fertility and extended with Beltsville Thawing Solution; Pursel and Johnson, 1975) at a concentration of 30×10^6/mL of spermatozoa. Semen from 4 different boars and doses was pooled and centrifuged ($1900 \times g$ for 3 minutes), the supernatant discarded, and the pellet resuspended to adjust the sperm concentration with phosphate-buffered saline (PBS) to 10×10^6/mL of spermatozoa.

Spermatozoa from a fertile Pietrain boar were cryopreserved as described by Roca et al (2003) in a plastic medium straw (0.5 mL). Spermatozoa were thawed in circulating water at 37°C for 20 seconds and washed 3 times by centrifugation at $1900 \times g$ for 3 minutes in Dulbecco PBS supplemented with 4 mg/mL of bovine serum albumin (BSA) (fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose, and 70 μg/mL of kanamycin. After washing, the sperm pellet was resuspended in PBS to a final concentration of 10×10^6/mL of spermatozoa.

Ten million spermatozoa (fresh or frozen-thawed) extended as

[1] All chemicals used in the preparation of the culture media were purchased from Sigma-Aldrich Co (Alcobendas, Madrid, Spain) unless otherwise stated.

described above were preincubated without (control) or with 1.5 mg/mL of PSP-I/PSP-II (from the same batch of lyophilized proteins) at 38°C. After 30 and 120 minutes of preincubation, sperm viability, motility, and acrosome status were evaluated.

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes Europe, Molecular Probes Europe, Leiden, The Netherlands). Briefly, 0.5 mL of sperm suspension containing 5×10^6 of fresh or frozen-thawed spermatozoa was stained with 25 nM SYBR-14 solution and 12 μM propidium iodide (PI) solution. Samples were incubated at room temperature in the dark for 10 minutes before cytometric analysis. All analyses were performed by analytical flow cytometry using an EPICS XL (Coulter Corporation Inc, Miami, Fla) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, Calif) with 15-mW laser power at 488 nm, and the software EXPO 2000 (Coulter Corporation). Forward and sideward light scatter were recorded for a total of 15 000 to 25 000 events per sample, and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis. SYBR-14 was measured through a 525-nm band pass filter, whereas PI was collected through a 635-nm band pass filter. Viable spermatozoa were defined as those stained with SYBR-14 and not stained with PI.

The percentage of motile spermatozoa was estimated with a computer-assisted motility analysis system. Sperm samples (5 μL of 10×10^6/mL of spermatozoa) were placed in a warm (38°C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38°C) of a Nikon Labophot (Kanagawa, Japan) equipped with a 10× contrast phase objective and a monochrome video camera (Hitachi CCD, Tokyo, Japan) connected to a personal computer. The sperm analysis was performed using the software Sperm Class Analyzer (Barcelona, Spain). The program settings were as follow: frames rate, 25 Hz; search radius, 11.5 μm; minimum track points, 7 frames; and threshold straightness, 75%. Two samples were examined with at least 100 spermatozoa being analyzed per sample.

Live spermatozoa showing acrosome exocytosis were evaluated using simultaneously PI to stain dead cells and the lectin FITC-PNA to evaluate disrupted acrosome. Briefly, 0.5 mL of sperm suspension containing 5×10^6 of fresh or frozen-thawed spermatozoa was stained with 12 μM of PI and 5 μL of FITC-PNA stock solution (1 mg/mL in bidistilled water). Spermatozoa were incubated for 5 minutes in the dark and analyzed immediately on the flow cytometer (see above). FITC-PNA was measured through a 530-nm band pass filter, whereas PI was measured through a 635-nm band pass filter. Spermatozoa were identified in 1 of the 3 following populations: PI positive, nonviable cells; PI negative and FITC-PNA negative, live spermatozoa with intact acrosome; and PI negative and FITC-PNA positive, corresponding to live spermatozoa with exocytosed acrosome.

Recovery, IVM, Sperm Penetration, and Evaluation of Oocytes

IM and IVM oocytes were used to assess sperm penetration ability (Martinez et al, 1993; Abeydeera and Day, 1997). Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 μg/mL of kanamycin maintained at 34°C to 37°C. Cumulus-oocyte complexes (COCs) were aspirated from medium-sized follicles (3 to 6 mm in diameter) with an 18-gauge needle fixed to a 10-mL disposable syringe. Only COCs with a compact cumulus mass (with at least 6 or 7 layers), an intact ZP, and an oocyte with an evenly granulated cytoplasm were selected for the different trials.

For preparation of IM oocytes, batches of 30 IM oocytes were placed directly in 2 mL of pre-equilibrated modified TCM-199 medium (Cheng, 1985) and kept in the incubator for approximately 30 minutes before spermatozoa were added for sperm penetration assays.

For preparation of IVM oocytes, the COCs were washed 3 times in BSA-free North Carolina State University (NCSU) 23 medium (Peters and Wells, 1993) supplemented with 10% (vol/vol) porcine follicular fluid, 0.1 mg/mL of cysteine, and 10 ng/mL of epidermal growth factor (IVM medium). Thereafter, COCs were transferred to a Nunc 4-well multidish plate (50 to 100 COCs per well) submerged in 500 μL of pre-equilibrated maturation medium (previously covered with warm mineral oil), supplemented with 10 IU/mL of eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/mL hCG (Intervet International BV), and cultured at 39°C in 5% CO_2 in air for 22 hours. The medium was then changed for maturation medium without hormone supplementation and incubated at 39°C in 5% CO_2 in air for another 22 hours. After IVM, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in IVM medium and washed 3 times with pre-equilibrated IVF medium (TBM medium as described by Abeydeera and Day, 1997). The latter consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2 · $2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine, and 0.2% BSA (fraction V; A 7888, initial fractionation by precipitation with cold alcohol). Batches of 50 IVM oocytes were placed in 50-μL drops of IVF medium covered with warm mineral oil in a 35 × 10-mm Petri dish. The dishes were kept in the incubator for approximately 30 minutes before spermatozoa were added for sperm penetration assays.

Spermatozoa exposed or not exposed to the PSP-I/PSP-II heterodimer, as described above, were centrifuged at 1000 × *g* for 3 minutes, and the sperm pellet was resuspended in modified TCM-199 medium for coincubation with IM oocytes or resuspended in IVF medium for coincubation with IVM oocytes. Spermatozoa were coincubated with IM or IVM oocytes at a oocyte-spermatozoa ratio of 1:66 000 (Martinez et al, 1993) or 1:2000 (Gil et al, 2004), respectively. The oocytes were coincubated with the spermatozoa at 39°C in an atmosphere of 5% CO_2 in air, the IM oocytes for 16 hours and the IVM oocytes for a primary period of 6 hours. The IVM oocytes were thereafter washed 3 times in pre-equilibrated embryo culture medium (NCSU-23 containing 0.4% BSA), transferred to a Nunc 4-well multidish containing 500 μL of the same medium per well (covered by mineral oil), and cultured for another 6 hours at 39°C and 5% CO_2 in air.

Following the coincubation, the IM oocytes were washed in PBS and repeatedly pipetted to mechanically remove the cumulus cells, and those spermatozoa attached to the surface of the ZP. The IM (16 hours post insemination) and IVM oocytes (12 hours post insemination) were mounted on slides, fixed, and stored in 25% (vol/vol) acetic acid in ethanol at room tempera-

ture for 48 to 72 hours, stained with 1% lacmoid in 45% (vol/vol) acetic acid, and examined under a phase contrast microscope at 400× magnification. Oocytes were considered penetrated when spermatozoa with intact (IM oocytes) or swollen heads and/or male pronuclei and their corresponding sperm tails (IVM oocytes) were found in the ooplasm.

The trials attempted to disclose the effect of the sperm exposition to PSP-I/PSP-II heterodimer on the ability of freshly diluted or frozen-thawed spermatozoa to penetrate pig oocytes (IM or IVM), being distributed as follows: 1) To study the penetrating ability of freshly extended boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IM oocytes, a total of 175 IM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 minutes at 38°C, whereas 182 IM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); 2) To study the penetrating ability of freshly extended boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IVM oocytes, a total of 400 IVM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 or 120 minutes at 38°C, whereas 415 IVM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); 3) To study the penetrating ability of frozen-thawed boar spermatozoa exposed to PSP-I/PSP-II heterodimer in IVM oocytes, a total of 792 IVM oocytes were cocultured with spermatozoa exposed to PSP-I/PSP-II (1.5 mg/mL in PBS) during 30 or 120 minutes at 38°C, whereas 773 IVM oocytes were cocultured with spermatozoa preincubated in PBS without the heterodimer (control); and 4) Finally, an experiment was designed, based on the results of experiment 3 with frozen-thawed spermatozoa, to evaluate the effect of sperm washing (by centrifugation in saline BSA or through Percoll gradient separation) to cleanse the sperm surface of frozen-thawed spermatozoa and, consequently, on the penetration capability of these spermatozoa on IVM oocytes. For this purpose, aliquots of 3 mL of re-extended postthaw semen (10×10^6/mL of spermatozoa in PBS) were preincubated for 2 hours at 38°C with 1.5 mg/mL of PSP-I/PSP-II heterodimer in PBS. Thereafter, spermatozoa were either washed 3 times by centrifugation at $1200 \times g$ for 3 minutes in 0.9% saline solution containing 1 mg/mL of BSA, with the final pellet being resuspended in fertilization medium at 2×10^6/mL of spermatoza, or centrifuged through a 35%/70% Percoll gradient ($900 \times g$ for 20 minutes), the lowest pellet being recovered and resuspended with fertilization medium at 2×10^6/mL of spermatozoa. Control groups were built by 1) spermatozoa preincubated in the presence of PSP-I/PSDP-II heterodimer as described above but pelleted without washing or 2) spermatozoa preincubated in the absence of PSP-I/PSP-II heterodimer. A total of 1592 IVM oocytes were cocultured with the spermatozoa as above described.

Statistical Analysis

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc, Chicago, Ill). Data were analyzed by analysis of variance (ANOVA) using the MIXED procedure according to a statistical model that included the fixed effects of presence of PSP-I/PSP-II heterodimer and incubation time and the random effect of replicate. In the last experiment, a washing procedure was included as a fixed effect. To analyze data of sperm viability, motility, and acrosome status, percentages were subjected to arcsine transformation before analysis. Data on the percentage of penetration and polyspermia were modeled according to the binomial model of parameters as described by Fisz (1980) before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be statistically significant when $P < .05$. Experiments were replicated 4 times.

Results

PSP-I/PSP-II Heterodimer Preserves the Viability and Motility of Freshly Extended and Frozen-Thawed Spermatozoa

Viability and motility percentages for freshly diluted spermatozoa before re-extension in PBS until 10×10^6/mL of spermatozoa were 95.2% ± 2.8% and 85.3% ± 2.8%, respectively. Likewise, viability and motility percentages for frozen-thawed spermatozoa before re-extension in PBS were 56.7% ± 3.2% and 45.7% ± 2.4%, respectively. The percentages of viable spermatozoa with an exocytosed acrosome were 2.1% ± 0.8% and 4.7% ± 1.4% for freshly extended and frozen-thawed spermatozoa, respectively. Exposition of spermatozoa to the PSP-I/PSP-II heterodimer had a significantly positive influence on sperm viability and motility for both freshly diluted and frozen-thawed spermatozoa (Figure 1A and B). Sperm viability and motility percentages were significantly higher for spermatozoa incubated with the PSP-I/PSP-II heterodimer than for spermatozoa incubated in the absence of the heterodimer. In relation to the spermatozoa with acrosome exocytosis, we have not noticed differences between spermatozoa exposed or unexposed to the PSP-I/PSP-II heterodimer (Figure 1C).

Preincubation of Freshly Extended Boar Spermatozoa With PSP-I/PSP-II Heterodimer Does Not Affect Their Ability to Penetrate IM Pig Oocytes

Results are presented in Table 1. Exposure of freshly diluted boar spermatozoa to PSP-I/PSP-II heterodimer did not influence either penetration or the number of spermatozoa per oocyte compared with controls.

Preincubation of Freshly Extended Boar Spermatozoa With PSP-I/PSP-II Heterodimer Does Not Affect Their Ability To Penetrate IVM Pig Oocytes

Results are presented in Table 2. Preincubation of freshly extended boar spermatozoa with 1.5 mg/mL of PSP-I/PSP-II heterodimer for 30 or 120 minutes did not significantly affect either penetration and polyspermy rates or the number of spermatozoa present in the ooplasm compared either with controls or over time.

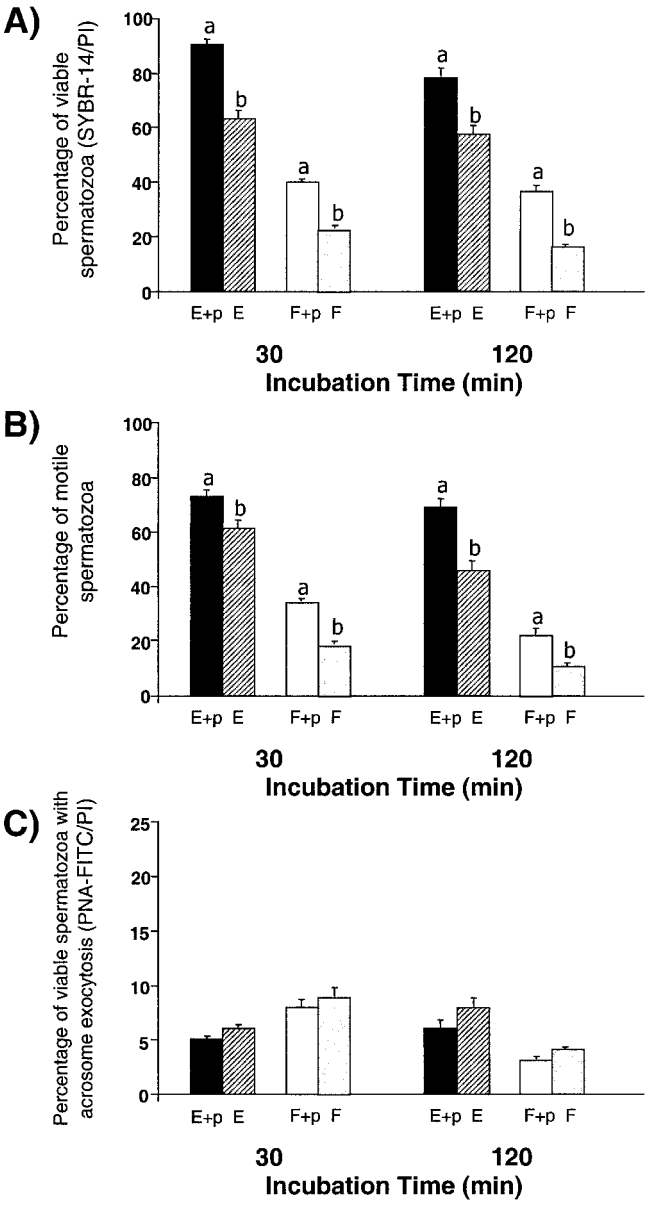

Figure 1. Effect of PSP-I/PSP-II heterodimer (1.5 mg/mL in phosphate-buffered saline) on the viability (A), motility (B), and acrosome exocytosis (C) of freshly extended (E+p) or frozen-thawed (F+p) boar spermatozoa extended until 10×10^6/mL of spermatozoa after 30 or 120 minutes of incubation at 38°C. Controls were treated with the same spermatozoa source but incubated in the absence of PSP-I/PSP-II heterodimer (E for extended and F for frozen-thawed spermatozoa). Columns represent the mean ± SEM (error bars) of 4 separate experiments. Different letters between columns per time and type of spermatozoa (freshly extended or frozen-thawed) indicate significantly different values ($P < .05$).

Preincubation of Frozen-Thawed Spermatozoa With PSP-I/PSP-II Heterodimer Decreases Their Ability to Penetrate IVM Pig Oocytes

Results are presented in Table 3. Control spermatozoa (preincubated in the absence of the PSP-I/PSP-II heterodimer) penetrated approximately 90% and 85% of oocytes at 30 and 120 minutes, respectively. The addition of PSP-I/PSP-II heterodimer at a final concentration of 1.5 mg/mL had a detrimental effect on the penetration rate at both 30 ($P < .05$) and 120 minutes ($P < .05$) compared with controls, indicating an immediate inhibitory effect that was maintained over time. Moreover, PSP-I/PSP-II also had an effect on the number of spermatozoa per oocyte, decreasing significantly ($P < .05$) with respect to controls and, consequently, increasing the percentages of monospermic oocytes.

Table 1. *Effect of preincubation of fresh boar spermatozoa with PSP-I/PSP-II heterodimer (1.5 mg/mL) on their ability to in vitro penetrate immature pig oocytes**

Group	Oocytes Penetrated (%)	Spermatozoa per Oocyte (No.)
Control (n = 182)	74.5 ± 3.5	6.4 ± 0.4
PSP-I/II exposed (n = 175)	72.8 ± 3.3	6.2 ± 0.2

* Data are expressed as mean ± SEM.

Sperm Cleansing (by Centrifugation in Saline BSA or Through Percoll Discontinuous Gradient Separation) of Frozen-Thawed Spermatozoa Previously Incubated With PSP-I/PSP-II Heterodimer Modifies Their Capability to Penetrate IVM Oocytes In Vitro

Table 4 displays the results from this experiment, showing that exposure of frozen-thawed boar spermatozoa to PSP-I/PSP-II significantly decreased penetration rates, polyspermy rates, and the number of spermatozoa per penetrated oocyte ($P < .05$). Washing (by centrifugation and re-extension in saline BSA) of pre-exposed spermatozoa further decreased penetration rates ($P < .05$) but did not influence the number of spermatozoa penetrated per oocyte, although the percentage of monospermic oocytes was high. On the other hand, the rate of oocyte penetration by spermatozoa cleansed by Percoll discontinuous gradient separation, albeit still lower than controls ($P < .05$), increased significantly ($P < .05$) compared with the internal control (exposed to PSP-I/PSP-II heterodimer but unwashed) or washing by centrifugation. No significant differences were seen for the number of spermatozoa per penetrated oocyte, which continued being fewer than in controls ($P < .05$), and the percentage of monospermic fertilization was, consequently, higher than in controls ($P < .05$).

Discussion

Biotechnological manipulation of semen (ie, flow cytometric sorting for chromosomal sex or cryopreservation) invariably induces changes in the viability, membrane in-

Table 2. *Effect of preincubation of fresh boar spermatozoa with PSP-I/PSP-II heterodimer (1.5 mg/mL) for 30 or 120 minutes on their ability to in vitro penetrate in vitro matured pig oocytes**

	Incubation Time (min)					
	30			120		
Group	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean Spermatozoa per Oocyte (No.)	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean Spermatozoa per Oocyte (No.)
Control (n = 202)	96.5 ± 1.2	95.3 ± 1.4	6.2 ± 0.4	98.4 ± 1.6	91.4 ± 1.1	7.4 ± 0.5
PSP-I/II exposed (n = 210)	97.1 ± 1.3	89.4 ± 1.2	5.3 ± 0.2	98.0 ± 1.4	90.5 ± 1.4	6.1 ± 0.3

* Data are expressed as mean ± SEM.

tegrity, acrosome status, and motility patterns of the spermatozoa (Maxwell et al, 1997; Maxwell and Johnson, 1997; Maxwell and Johnson, 1999). Attempts to minimize these effects include the addition of a certain proportion of whole (homologous or autologous) SP in the medium where spermatozoa are extended or simply bathe in. However, the high variability in the composition of the SP among males of the same species and among ejaculates of a single male prompted us to evaluate a specific protein, the spermadhesin PSP-I/PSP-II heterodimer, as an additive to protect the spermatozoa against the detrimental effect of the above mentioned biotechnological manipulations. To avoid variability among samples, the experiments were performed using either pools of ejaculates from several artificially inseminated boars or frozen-thawed spermatozoa from a single ejaculate from a fertile artificially inseminated boar.

The experiments were performed at a concentration of 1.5 mg/mL of PSP-I/PSP-II, which represents the concentration of this particular spermadhesin in 10% of bulk SP (Dostálová et al, 1994; Calvete et al, 1995a). At this concentration, the PSP-I/PSP-II has the sperm protective effect of SP concentration (10%) (Maxwell et al, 1997) as observed previously (Centurión et al, 2003).

Under the present experimental conditions, exposure to the heterodimer dramatically improved sperm viability in vitro, thus confirming the above mentioned previous results. This pretreatment did not, however, affect sperm penetration rates or sperm numbers per oocyte when pretreated fresh spermatozoa were coincubated with IM or IVM oocytes; that is, no particular beneficial effect was seen in fertilization rates. Rates of polyspermy were not affected when IVM oocytes were evaluated. Polyspermy rate was not evaluated in IM oocytes, because they lack the mechanism for sperm penetration block.

By contrast, when cryopreserved spermatozoa were tested for penetration of IVM oocytes, a 30-minute exposure to PSP-I/PSP-II during pre-IVF incubation showed a significant blocking effect on penetration rate and on mean sperm numbers per oocyte and decreasing polyspermy. This effect lasted for 120 minutes before oocyte exposure. This unexpected result motivated a cleansing of the spermatozoa by centrifugation in saline BSA or through Percoll density discontinuous gradient separation before repeating the IVF procedure. Penetration rate (but not the number of spermatozoa per oocyte) significantly increased when spermatozoa were filtered through Percoll compared with either washed spermatozoa or unwashed

Table 3. *Effect of preincubation of frozen-thawed boar spermatozoa (single batch) with PSP-I/PSP-II heterodimer (1.5 mg/mL) for 30 or 120 minutes at 38°C on their ability to in vitro penetrate in vitro matured pig oocytes**

	Incubation Time (min)					
	30			120		
Group	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean Spermatozoa per Oocyte (No.)	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean Spermatozoa per Oocyte (No.)
Control (n = 390)	90.5 ± 1.5 A†	73.6 ± 1.9 A	2.9 ± 0.2 A	85.3 ± 1.8 A	64.2 ± 1.9 A	2.6 ± 0.3 A
PSP-I/II exposed (n = 402)	32.3 ± 2.3 B	41.2 ± 2.8 B	1.6 ± 0.1 B	25.6 ± 2.2 B	35.7 ± 2.3 B	1.4 ± 0.1 B

* Data are expressed as mean ± SEM.
† Different letters in the same column indicate significantly different values ($P < .05$).

Table 4. *Effect of sperm cleansing (by centrifugation in saline bovine serumalbumin or through discontinuous Percoll gradient separation) of frozen-thawed spermatozoa (single batch) previously incubated for 120 minutes at 38°C with PSP-I/PSP-II heterodimer (1.5 mg/mL) on their capability to in vitro penetrate in vitro matured oocytes**

Group	Oocytes Penetrated (%)	Oocytes Polispermic (%)	Mean Spermatozoa per Oocyte (No.)
Control (n = 402)	91.1 ± 2.2 A†	68.7 ± 1.9 A	2.6 ± 0.2 A
Exposed to PSP-I/II (n = 395)	30.8 ± 3.6 B	36.4 ± 2.7 B	1.4 ± 0.1 B
Exposed to PSP-I/II and washed by centrifugation (n = 390)	12.8 ± 1.8 C	25.1 ± 2.3 C	1.6 ± 0.1 B
Exposed to PSP-I/II and cleansed by Percoll gradient centrifugation (n = 405)	53.1 ± 4.2 D	38.5 ± 2.9 B	1.4 ± 0.2 B

* Data are expressed as mean ± SEM.
† Different letters in the same column indicate significantly different values ($P < .05$).

controls. The Percoll treatment yielded a significant decrease in polyspermy.

The blockade of sperm penetration by incubation with the heterodimer seemed to be a rather rapid phenomenon, since the number of oocytes penetrated with spermatozoa decreased from 90% to 33% of oocytes when the PSP-I/PSP-II heterodimer was added to the sperm suspension just before coincubation with the oocytes (30 minutes). Cleansing seemed to improve this situation, suggesting that the cryopreservation procedure might cause a coating effect by the extender components on the surface of the spermatozoa.

Such results are puzzling for several reasons. PSP-I/PSP-II heterodimer is the major protein in the sperm-rich fraction of the ejaculate (Centurión et al, 2003). Boar spermatozoa preincubated with SP from selected portions of the sperm-rich fraction, where low doses of the PSP-I/PSP-II heterodimer (in the range of the ones used hereby) are present, sustained in vitro manipulation (such as extension, storage, or cryopreservation) better (Peña et al, 2003). Also, boar spermatozoa preincubated with SP from the sperm-rich fraction produced better IVF results than when spermatozoa were incubated with SP from other fractions of the ejaculate (Zhu et al, 2000).

The PSP-I/PSP-II heterodimer seems to influence the ZP and decrease sperm penetration in vitro. Exposure of pig oocytes to 1.5 mg/mL of PSP-I/PSP-II heterodimer in the IVF medium was able to significantly decrease penetration rates by untreated boar spermatozoa (Caballero et al, unpublished data) either by blocking sperm ZP receptors or acting on the surrounding spermatozoa during IVF. Such a response is unlikely to occur in vivo, since PSP-I/PSP-II does not seem to maintain attachment to the ZP following sperm transport in the female genital tract (as other SP proteins, such as the AWN, do; Rodriguez-Martinez et al, 1998). The PSP-I/PSP-II heterodimer seems to be easily removed from the sperm surface by in vitro capacitation treatments (Calvete et al, 1995a). Ejaculated spermatozoa (surrounded by SP) can undergo capacitation and acrosome reaction when coincubated with oocytes, even when not washed or preincubated in capacitating media (Martinez et al, 1996).

Pre-exposure of spermatozoa to the heterodimer should not affect the interaction between spermatozoa and oocytes, thus explaining why no positive effects were seen when freshly extended spermatozoa were tested with IM or IVM oocytes. On the other hand, there is no easy explanation as to why frozen-thawed spermatozoa could be influenced so negatively by the heterodimer when no detrimental effects on sperm viability, motility, and acrosome were recorded (experiment 1). To the best of our knowledge, no data concerning interactions between PSP-I/PSP-II and cryopreserved spermatozoa are available. This calls for further studies, particularly in light of a lack of relationship among normal levels of sperm viability, their ability to acrosome react in vitro, and their penetrating capacity on in vivo matured oocytes (Vazquez et al, 1993).

Several experiments have shown that inseminating boar spermatozoa, either cryopreserved or sex selected (by flow cytometry sorting), results in lower farrowing rates and decreased litter size when compared with freshly ejaculated and liquid extended spermatozoa (Roca et al, 2003; Vazquez et al, 2003). This decrease in pregnancy rates is considered to be due to ''capacitation-like'' alterations in the plasma membrane and changes in motility patterns (similar to hyperactivated motility) caused by cooling or flow cytometry sorting. These alterations shorten the life span of those spermatozoa that survive these manipulations by provoking premature spontaneous acrosome exocytoses and cell death (Maxwell and Johnson, 1997; Green and Watson, 2001; Kaneto et al, 2002).

The status of the plasma membrane is altered in manipulated spermatozoa, and the exposure to PSP-I/PSP-II

heterodimer increases the life span of highly extended boar spermatozoa (Centurión et al, 2003). We argue that the PSP-I/PSP-II protective effect on the spermatozoa may be linked to its interacting capability with the plasma membrane of ''capacitated-like'' spermatozoa, which in turn impairs the ability of the spermatozoa to penetrate the oocyte vestments.

As mentioned above, removal of SP spermadhesin occurs during in vivo sperm transport through the female genital tract (Calvete et al, 1997; Mortimer, 2000), and only very small concentrations of some spermadhesins (such as the AWN) are present on the sperm plasmalemma when reaching the ZP (Rodriguez-Martinez et al, 1998). This removal of SP components could be the reason for the partial restoration of the ability to penetrate oocytes by Percoll cleansed frozen-thawed spermatozoa. This procedure is more effective for sperm cleansing that any other available procedure (Rodriguez-Martinez et al, 1997).

Our results are also in line with previous investigations that show that sperm washings by centrifugation in saline BSA can damage the spermatozoa (Harrison and White, 1972) and, consequently, lead to a decrease in penetration rates when used for IVF (Martinez et al, 1996). This effect may be amplified when ''weaker'' spermatozoa, such as those cryopreserved or flow cytometry–sorted spermatozoa, are washed by centrifugation in saline BSA. Consequently, washing by centrifugation in saline BSA may not be the most appropriate method to restore the fertilizing ability of spermatozoa preincubated with PSP-I/PSP-II. In contrast, discontinuous density gradient centrifugation with Percoll seemed effective to restore the fertilizing capability of some of the spermatozoa. Our results are in agreement with reports of the enhancement of penetration rates of Percoll-washed boar spermatozoa (Grant et al, 1994; Jeong and Yang, 2001). Moreover, using this cleansing method, at higher monospermic penetration was observed, although further studies are necessary, particularly in light of the correlation established between the absolute number of spermatozoa penetrated per oocyte and the degree of oocytes penetrated and polyspermy (Rath, 1992; Gil et al, 2004). Although washing by centrifugation in saline removes bulk SP from spermatozoa more rapidly than Percoll separation (Levay et al, 1995), the latter cleanses the surface without damaging the plasmalemma structure, thus maintaining sperm viability (Rodriguez-Martinez et al, 1997). Most spermatozoa were recovered in the soft pellet at the bottom, and only a very low percentage of the spermatozoa were recovered at the interface of the 35% and 70% Percoll layers, which may indicate that the action of Percoll on the spermatozoa was more associated with a ''cleansing effect'' than with an effect related to selection and enrichment of the population recovered. However, whether the effect of Percoll procedure is only due to a cleansing effect remains to be clarified.

In conclusion, the results confirm that short-time exposure of fresh or frozen-thawed boar spermatozoa to low doses of the seminal heterodimer PSP-I/PSP-II preserves (or improves) sperm viability and motility in vitro without affecting the sperm acrosome. Although there is no obvious influence of the heterodimer on the capability of fresh extended boar spermatozoa to penetrate homologous oocytes (either IM or IVM), PSP-I/II exerts a deleterious effect when frozen-thawed spermatozoa are used to penetrate IVM oocytes. However, this inhibition did not seem permanent, since a subsequent washing through a Percoll gradient restored sperm function in some of the cells. We are currently investigating the mechanism by which purified PSP-I/PSP-II modulates sperm viability and oocyte penetration ability in vitro and the effect of this protein on in vivo fertilizing capacity in pigs.

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