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Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction

Ignacio Caballero[1], *Juan M. Vázquez*[1], *Heriberto Rodríguez-Martínez*[1,2], *Maria A. Gil*[1], *Juan J. Calvete*[3], *Libia Sanz*[3], *Eva M. Garcia*[1], *Jordi Roca*[1] *and Emilio A. Martínez*[1]
Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain; Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden; and Institute of Biomedicine, CSIC, Valencia, Spain

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Summary

The seminal plasma PSP-I/PSP-II spermadhesin is able to preserve, *in vitro*, the viability of highly extended boar spermatozoa, suggesting it might be used as a suitable ameliorator for the damaging effects of sperm handling, including *in vitro* fertilization. However, little is known about the ligand capability of PSP-I/PSP-II as regards the zona pellucida (ZP) or its possible role in gamete interaction. The present study evaluated the effect of the presence of PSP-I/PSP-II (1.5 mg/ml) during *in vitro* oocyte maturation and also during co-incubation of frozen-thawed boar spermatozoa with either immature (IM) or *in vitro* matured (IVM) oocytes, either enclosed by cumulus cells or denuded. Exposure of the gametes to the heterodimer during *in vitro* gamete co-incubation showed a significant blocking effect of sperm penetration rates and a decreased number of spermatozoa per oocyte in both IM and IVM denuded oocytes. Such an effect was not present in cumulus-enclosed oocytes, suggesting the effect could be mediated by exposed ZP receptors. In addition, when PSP-I/PSP-II was added to the IVM medium, oocyte maturation rates were significantly reduced. In conclusion, the results suggest that PSP-I/PSP-II, when present *in vitro*, blocks sperm–ZP binding.

Keywords: Gametes, IVF, Pig, PSP-I/PSP-II, Seminal plasma

Introduction

Gamete interaction is an early critical step in mammalian fertilization, involving at least three morphologically disparate cell types: the capacitated acrosome-intact spermatozoon, the mature oocyte and the surrounding cumulus cells (Yanagimachi, 1994). The events of gamete recognition, binding and fusion are highly regulated processes that imply a number of biochemical reactions until a new zygote is formed. In domestic species, this mechanism of cell-to-cell adhesion seems to be mediated by protein–carbohydrate interactions between sperm-associated lectins and glycan structures of the oocyte zona pellucida (ZP), the latter synthesized by the concerted action of the oocyte and the granulosa cells during oocyte maturation (Sinowatz *et al.*, 2001).

One of these sperm lectins constitutes the spermadhesin family (Sinowatz *et al.*, 1997). Boar spermadhesins are a group of (glyco)proteins built by a single CUB domain architecture (Romero *et al.*, 1997), coating the sperm surface (Dostálová *et al.*, 1994). They play a role in several biological functions, including sperm capacitation and, as already mentioned, gamete recognition and binding (Calvete *et al.*, 1994). According to their binding properties, spermadhesins can be divided into two 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*, the membrane integrity, motility and mitochondrial activity of highly extended spermatozoa for as long these are exposed to the heterodimer (Centurión

Correspondence to: J.M. Vázquez, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain. Tel: +34 968 364736. Fax: +34 968 367069. e-mail: vazquez@um.es
[1] Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain.
[2] Department of Obstetrics & Gynaecology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.
[3] Institute of Biomedicine, CSIC, Valencia, Spain.

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et al., 2003). Spermadhesin PSP-I/PSP-II reproduces the protective effect that has been reported upon the addition to spermatozoa of either bulk seminal plasma (SP) from selected males (Maxwell *et al*., 1997; Maxwell & Johnson, 1999; Caballero *et al*., 2004) or SP components of low molecular weight (Ashworth *et al*., 1994). These properties highlight PSP-I/PSP-II as a potential candidate for pre-treatment of manipulated spermatozoa (extended, stored, deep-frozen or sex-sorted) with the aim of promoting sperm survival and performance *in vitro*. However, the competence of PSP-I/PSP-II to interact during gamete binding is still unclear. While the heparin-binding spermadhesin AWN is able to reach the ovulated oocyte(s) *in vivo* (Rodríguez-Martínez *et al*., 1998), the PSP-I/PSP-II heterodimer binds loosely to the sperm surface and does not seem to maintain any attachment to the spermatozoa following either *in vitro* capacitation or sperm transport in the female genital tract, as is seen to be the case with the AWNs (Dostálová *et al*., 1994; Calvete *et al*., 1997; Rodríguez-Martínez *et al*., 1998). In some studies, PSP-I/PSP-II heterodimer showed affinity for ZP glycoproteins and this activity was located in the isolated PSP-II monomer and was reported to be cryptic in the heterodimer (Calvete *et al*., 1995*a*). In contrast, Jonáková *et al*. (2000) also found an interaction between the ZP and isolated PSP-II, but this binding was inhibited by the aggregated PSP-I/PSP-II heterodimer. Therefore, although an *in vivo* functional role of this spermadhesin during gamete binding is unexpected, the fact that the ZP-binding properties of PSP-I/PSP-II heterodimer are still a matter of controversy in the literature calls for further research in this area, if the aim is to use these spermadhesins during *in vitro* gamete handling, for instance as a preparatory step for IVF.

Consideration of the beneficial effect on sperm viability already assessed *in vitro* and the divergent results regarding the ZP-binding ability of PSP-I/PSP-II, has prompted us to examine the effect of the PSP-I/PSP-II heterodimer during boar sperm–oocyte co-culture using both immature (IM) and *in vitro* matured (IVM) homologous oocytes.

Materials and methods

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

Isolation of spermadhesin PSP-I/PSP-II heterodimer from boar seminal plasma

All experiments were performed with the SP from sexually mature boars which had previously sired offspring. Ejaculates were obtained using the gloved-hand method and SP was separated from spermatozoa by centrifugation at 3800 *g* for 15 min, 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, 0.025% sodium azide, pH 7.4 (Calvete *et al*., 1995*a*). The identity and purity of the protein preparation was assessed by N-terminal sequence analysis (using an Applied Biosystems 472 automated protein sequencer, Langen, Germany) and MALDI-TOF mass spectrometry (using an Applied Biosystems Voyager DE-Pro mass spectrometer, Langen, Germany). 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 (27332 M^{-1} cm^{-1}) determined by Menéndez *et al*. (1995), or by amino acid analysis (after sample hydrolysis in 6 M HCl for 24 h at 106 °C in evacuated and sealed ampoules) using a Beckman Gold Amino Acid Analyser (Beckman, Barcelona, Spain). Proteins were dialysed against distilled water, lyophilized, and stored at −20 °C until used.

Culture media

The basic medium used to assess *in vitro* the sperm penetration ability of immature oocytes was TCM-199 with Earle's salts supplemented with 12% heated fetal calf serum (v/v), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 IU penicillin G, and 30 μg/ml streptomycin sulfate (Cheng *et al*., 1986).

The basic medium used for *in vitro* maturation was bovine serum albumin (BSA)-free North Carolina State University (NCSU) 23 medium (Peters & Wells, 1993) supplemented with 10% (v:v) porcine follicular fluid, 0.1 mg/ml cysteine and 10 ng/ml epidermal growth factor (EGF). This medium is called 'IVM medium' hereafter.

The *in vitro* fertilization (IVF) medium used (Abeydeera & Day, 1997) consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2{\cdot}2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine and 0.2% BSA (fraction V, Cat. no. A 7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was NCSU 23 containing 0.4% BSA (fraction V, Cat no. A 8022, initial fractionation by cold alcohol precipitation).

Recovery, *in vitro* maturation, penetration and evaluation of oocytes

IM and IVM oocytes were used to assess sperm penetration ability (Martínez *et al*., 1993; Abeydeera & Day, 1997).

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Ovaries were obtained from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 μg/ml kanamycin, maintained at 34–37 °C. Cumulus–oocyte complexes (COCs) were aspirated from medium-sized follicles (3–6 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 six or seven layers), an intact ZP and an oocyte with evenly granulated cytoplasm were selected for the different experiments.

For preparation of IM oocytes, oocytes were divided in two groups of cumulus-enclosed (CE) and denuded oocytes, respectively. Both CE oocytes and those denuded by repeated pipetted to mechanically remove the cumulus cells, were placed directly in 2 ml of pre-equilibrated modified TCM-199 medium supplemented with 2 mM caffeine and 5.4 mM calcium lactate (Cheng *et al*., 1986) in batches of 30 IM oocytes and kept in the incubator at 39 °C in 5% CO_2 in air for about 30 min before spermatozoa were added for sperm penetration assays.

For preparation of IVM oocytes, the COCs were washed three times in IVM medium. Thereafter, COCs were transferred to a Nunc 4-well multidish plate (50–100 COCs per well) submerged in 500 μl of pre-equilibrated maturation medium (previously covered with warm mineral oil), supplemented with 10 IU/ml eCG (Intervet International, Boxmeer, The Netherlands) and 10 IU/ml hCG (Intervet International, Boxmeer, The Netherlands), and cultured at 39 °C in 5% CO_2 in air for 22 h. 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 h. After *in vitro* maturation, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in IVM medium and washed three times with pre-equilibrated IVF medium (TBM medium as described by Abeydeera & Day, 1997). 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 about 30 min before spermatozoa were added for sperm penetration assays.

Frozen-thawed spermatozoa were cryopreserved from a fertile Pietrain boar as described by Roca *et al*. (2003) in a plastic medium straw (0.5 ml) and thawed in circulating water at 37 °C for 20 s. Thawed spermatozoa were re-suspended in modified TCM-199 for co-incubation with IM oocytes. For IVM oocytes, thawed semen was washed three times by centrifugation at 1900 *g* for 3 min in Dulbecco's phosphate-buffered saline (PBS) supplemented with 4 mg/ml BSA (Fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 μg/ml kanamycin (mDPBS). Spermatozoa were co-incubated with IM or IVM oocytes at a oocyte:spermatozoa ratio of 1:66 000 (Martínez *et al*., 1993) or 1:2000 (Gil *et al*., 2004), respectively. The oocytes were co-incubated with the spermatozoa at 39 °C in an atmosphere of 5% CO_2 in air, the IM oocytes for 16 h and the IVM oocytes for a primary period of 6 h. The IVM oocytes were thereafter washed three 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 h at 39 °C and 5% CO_2 in air.

Following the co-incubation, IM oocytes were washed in PBS and repeatedly pipetted to mechanically remove the cumulus cells (group of CE oocytes) and those spermatozoa attached to the surface of the ZP (groups of CE and denuded oocytes). The IM (16 h post-insemination) and the IVM oocytes (12 h post-insemination) were mounted on slides, fixed and stored in 25% (v/v) acetic acid in ethanol at room temperature for 48–72 h, stained with 1% lacmoid in 45% (v/v) acetic acid, and examined under a phase-contrast microscope at ×400 magnification. Oocytes were considered penetrated when spermatozoa with unswollen (IM oocytes) or swollen heads and/or male pronuclei and their corresponding sperm tails (IVM oocytes) were found in the ooplasm.

Experimental design

Experiments were designed to disclose the effect of the presence of PSP-I/PSP-II heterodimer (1.5 mg/ml) during sperm–oocyte co-incubation on penetration rates and number of spermatozoa per oocyte in IM (experiment 1, including a total of 673 IM oocytes) or IVM oocytes (experiment 2, including a total of 730 IVM oocytes). All experiments were carried out with the same batch of lyophilized proteins. In addition, frozen-thawed spermatozoa from a single ejaculate were used to avoid inter-ejaculate variability. In order to rule out confounding effects related to the presence of cumulus cells, CE and denuded IM oocytes were exposed to the heterodimer during co-incubation with frozen-thawed boar spermatozoa (single batch) in a 2 × 2 factorial design. Controls were provided by PSP-I/PSP-II unexposed, co-incubated gametes. All experiments were replicated three times.

Data analysis

All the data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS, Chicago, IL). Data were analysed by ANOVA using the MIXED procedure according to a statistical model including the fixed effect of the presence of PSP-I/PSP-II heterodimer and the random effect of replicate. For experiment 1, presence or absence of CE was included as fixed effect and interaction with the heterodimer PSP-I/PSP-II was considered. Data on percentage of penetration were modelled according to the binomial model of parameters as described by Fisz (1980) before analysis.

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Table 1 Effect of the presence of PSP-I/PSP-II heterodimer (1.5 mg/ml) during co-culture of sperm with either cumulus-enclosed (CE) or cumulus-denuded immature (IM) pig oocytes on the percentage of oocytes penetrated and the number of spermatozoa per oocyte

PSP-I/PSP-II	Type of immature oocyte	No. of oocytes	% of oocytes penetrated	No. of spermatozoa per oocyte
Unexposed	CE	196	79.0 ± 2.9^a	8.5 ± 0.5^a
	Denuded	151	95.0 ± 1.8^b	20.4 ± 0.9^b
Exposed	CE	194	72.0 ± 3.3^a	7.4 ± 0.6^a
	Denuded	132	67.0 ± 4.3^a	12.9 ± 1.1^c
Probability				
PSP-I/PSP-II			0.001	0.001
Type of immature oocyte			NS	0.001
PSP-IPSP-II × Type of immature oocyte			0.001	0.004

Values are the mean ± SEM.
Values within columns with different superscripts differ significantly ($p < 0.05$).

When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $p < 0.05$.

Results

Experiment 1: Effect of adding 1.5 mg/ml of PSP-I/PSP-II heterodimer to the sperm–IM oocyte co-culture medium on sperm penetration ability

Results are presented in Table 1. The presence of the cumulus cells affected the number of spermatozoa per oocyte ($p < 0.05$) while the presence of the heterodimer PSP-I/PSP-II affected both penetration rates and number of spermatozoa per oocyte ($p < 0.05$). The interaction between the presence of cumulus cells and the heterodimer was significant ($p < 0.05$) for both penetration rates and number of spermatozoa per oocyte. In control groups (unexposed), a significant increase in sperm penetration rates and number of spermatozoa per oocyte was found in denuded IM oocytes compared with CE IM oocytes ($p < 0.05$). When both types of IM oocytes were exposed to PSP-I/PSP-II, only the denuded oocytes showed a significant difference compared with controls, the sperm penetration rates and number of spermatozoa per oocyte becoming significantly lower ($p < 0.05$).

Experiment 2: Effect of adding 1.5 mg/ml of PSP-I/PSP-II heterodimer to the sperm–IVM oocyte co-culture medium on sperm penetration ability

Results are presented in Table 2. Co-culture of sperm–IVM oocytes in IVF medium supplemented with 1.5 mg/ml of PSP-I/PSP-II heterodimer blocked the penetration of spermatozoa into the oocytes, resulting in a significant decrease in both penetration rates and number of spermatozoa per oocyte compared with controls ($p < 0.05$).

Table 2 Effect of the presence of PSP-I/PSP-II heterodimer (1.5 mg/ml) during co-culture of sperm and in vitro matured (IVM) oocytes in IVF medium on the percentage of oocytes penetrated and the number of spermatozoa per oocyte

Group	No. of oocytes	Penetration rate (%)	No. of spermatozoa per oocyte (mean)
Control	348	98 ± 0.7^a	6.7 ± 0.2^a
PSP-I/PSP-II-exposed	382	46 ± 2.8^b	1.9 ± 0.8^b

Values are the mean ± SEM.
Values within columns with different superscripts differ significantly ($p < 0.05$).

Discussion

The addition of small amounts of the spermadhesin PSP-I/PSP-II (1.5 mg/ml), which have proven protective for boar sperm viability (Centurión *et al*., 2003), to denuded-IM oocytes or IVM oocytes co-cultured with frozen-thawed boar spermatozoa induced a significant decrease of both *in vitro* penetration rates and mean numbers of spermatozoa per oocyte. Boar SP contains many proteins that have affinity for the ZP (Peterson *et al*., 1989; Jonáková *et al*., 2000), inhibiting the binding of spermatozoa. The ability of the PSP-I/PSP-II heterodimer and its isolated subunits (PSP-I and PSP-II) to bind known ligands has been previously reported with contradictory results. While PSP-I does not bind to ZP using a biotinylated ligand assay (Calvete *et al*., 1995*a*), the PSP-II subunit shows affinity for ZP glycoproteins (Calvete *et al*., 1995*a*; Jonáková *et al*., 2000). Furthermore, some the discrepancies include the affinity of the aggregated PSP-I/PSP-II heterodimer for the ZP

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(Calvete *et al*., 1995*a*; Jonáková *et al*., 2000). In other words, the degree of interaction between the oocyte vestment and PSP-I/PSP-II is far from clear.

Variations in the amount and distribution of the ZP (glyco)components of many species during oocyte growth have been reported (Parillo & Verini Supplizi 1999; Parillo *et al*., 2001). In a previous experiment (data not shown), exposure of COCs to PSP-I/PSP-II inhibited nuclear maturation, with only 32% of the oocytes exposed to PSP-I/PSP-II reaching metaphase II while 84% of the unexposed oocytes matured normally. Moreover, the expansion of cumulus cells was virtually inhibited in a large proportion of these PSP-I/PSP-II-exposed COCs. The fact that the IM oocyte population collected from abattoir ovaries is heterogeneous in quality and size (Roca *et al*., 1998; Lucas *et al*., 2002) could explain why PSP-I/PSP-II exposure did not affect the entire oocyte population, since the oocytes could have been in different growth stages. Growing oocytes would then be surrounded by different glycoprotein domains, as these are sequentially synthesized and added by the follicular cells and the oocyte or are modified in the ZP along with follicular growth (Lucas *et al*., 2003); either one of these factors could result in modifications of the affinity of the ZP for the heterodimer PSP-I/PSP-II.

The mechanism by which the heterodimer PSP-I/PSP-II mediates *in vitro* maturation might be different from the effect it exerts during *in vitro* sperm penetration of porcine oocytes. Therefore, the effect of PSP-I/PSP-II on the penetration ability of spermatozoa could be unrelated to the blockade of ZP binding by the heterodimer, since whole SP and some of its components have already shown inhibitory effects on sperm–oocyte binding by deterring the functionality of the spermatozoa before they bind to the ZP (Peterson *et al*., 1984; Capková & Peknicová 1997).

While the presence of cumulus cells exerted a detrimental effect on sperm penetration rates in co-culture of sperm and IM oocytes not exposed to PSP-I/PSP-II, the cumulus cells surrounding the oocytes elicited a beneficial effect on the *in vitro* penetration ability of the spermatozoa when heterodimer PSP-I/PSP-II was present. Although easy removal of the heterodimer from the sperm surface was expected to occur *in vitro* (Calvete *et al*., 1995*b*), similar rates between control and treatment arms of the experiment were seen only when IM oocytes surrounded by cumulus cells were co-incubated with spermatozoa in the presence of PSP-I/PSP-II. Penetration of spermatozoa through the cumulus oophorus cloud, a structure composed not only of the cumulus cells but primarily by the glycosaminoglycan-based fibrous matrix they secrete, made up of hyaluronic acid and proteoglycans, is considered to facilitate sperm capacitation and penetration (Fléchon *et al*., 2003). Whether this passage through the cumulus cloud implies that the carbohydrate of the proteoglycan matrix would trap PSP-I/PSP-II heterodimer, thus cleansing the sperm surface of attached SP proteins, or whether the heterodimer is removed during sperm plasma membrane redistribution during sperm capacitation in the presence of cumulus cells, is as yet unknown. However, these are tempting hypotheses to explain why higher oocyte penetration rates following exposure to PSP-I/PSP-II were found in cumulus-enclosed IM oocytes.

In conclusion, while there is no obvious influence of the PSP-I/PSP-II heterodimer on the co-culture of CE oocytes and spermatozoa, a deleterious effect is observed in the absence of cumulus cells in both IM and IVM oocytes. Whether this inhibitory effect is exerted directly on the spermatozoa or by blocking ZP receptors is unknown. Further research is therefore needed to clarify the mechanisms by which the PSP-I/PSP-II heterodimer affects oocyte–sperm interaction.

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