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Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow

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Short title: PSPs are chemoattractant for post-mating inflammation in pigs

Key words: seminal plasma, spermadhesins, PMNs, inflammation, T cells, endometrium, *in vivo*, pig.

Abstract

The heterogeneous seminal plasma (SP) is involved in endometrial inflammation and following immune changes that occur post-mating in pigs. This study tested whether SP spermadhesins (binding heparin [HBPs] or not [PSP-I/PSP-II]) recruit different lymphocyte subsets ($CD2^+$, $CD4^+$ and $CD8^+$) and polymorphonuclear leukocytes (PMNs) to the superficial endometrium/lining epithelium and lumen, respectively, of oestrous sows, *in vivo*. In Experiment 1, endometrial biopsies were taken under narcosis from six sows whose uterine horns were separated and their lumen infused with 3 mg/mL spermadhesins (HBP resp PSP-I/PSP-II) in 100 mL saline (treatment horn) or 100 mL saline (control horn). Endometrial samples removed between 2 and 120 min post-infusion were studied by immunohistochemistry (IHC, using mABs) or histology, to assess the degree of T-cell respectively PMN entry over time. In Experiment 2, eight conscious oestrous sows were singly infused intra-utero with 3 mg/mL of PSP-I/PSP-II in 100 mL saline ($n = 4$) or 100 mL of saline ($n = 4$), to assess relative PMN numbers in the uterine lumen, 3 h post-infusion. Compared to controls, PSP-I/PSP-II infusion significantly recruited uterine lymphocytes from 10 min ($CD2^+$) or from 60 min ($CD8^+$) onwards, while HBPs had only increased $CD4^+$ cells by 120 min. As well, PSP-I/PSP-II, but not HBPs, significantly ($P < 0.05$) induced an early (10 min) PMN migration to the surface epithelium, 5-fold by 30 min and 7-fold from 60 min onwards ($P < 0.001$), with PMNs visible in the lumen from 30 min of infusion. Six-fold more PMNs were collected from the uterine lumen of PSP-I/PSP-II-infused sows compared to controls, 3 h after infusion ($P < 0.001$). Seminal plasma PSP-I/PSP-II heterodimer triggers the entry of cleansing uterine PMNs, initiating a cascade of transient and long-lasting immunological events in oestrous sows.

1.1 Introduction

In the pig, as in many other species, mating causes a transient endometrial inflammation (reviewed by Robertson et al., 2006) which, at first sight, is a logical step by the female genital tract to combat the entry of foreign cells (e.g. the spermatozoa), foreign proteins (in the seminal plasma, SP) and, eventually, of pathogens. Some spermatozoa escape this inflammation by reaching the oviductal sperm reservoir (SR) and participating in fertilization. Spermatozoa, and later the early embryo, the foetus and the placenta, are immuno-tolerated by

the female, despite all being allogeneic (they contain transcripts of paternal origin) and thus liable to immune rejection. How is this accomplished?

The boar ejaculate is large and fractionated; most spermatozoa are delivered in a sperm-rich fraction (SRF) followed by a post-SRF containing increasing amounts of SP proteins (reviewed by Rodriguez-Martinez et al., 2005). Shortly after sperm deposition, a certain proportion of the spermatozoa colonize the tubal SR, whose pre-ovulatory functionality ensures their viability and potential fertilizing capacity in an inflammatory-free environment (reviewed by Rodriguez-Martinez, 2007). The other spermatozoa, the major proportion, are removed from the uterine cavity by retrograde flow (20–25% of the spermatozoa leaving within 30 min, Steverink et al., 1998), while the rest are trapped (neutrophil extracellular traps, [NETs], Alghamdi et al., 2009) and phagocytosed by invading polymorphonuclear granulocytes (PMNs), which apparently start entering the lumen ~10 min after artificial insemination (AI), peak entry by 30 min and continue to enter for the following 2–3 h (Lovell and Getty, 1968; Viring and Einarsson, 1981; Rodriguez-Martinez et al., 1990), largely exceeding the number of inseminated spermatozoa (Matthijs et al., 2003). This uterine PMN influx is accompanied by accumulation of macrophages, granulocytes and lymphocytes in the endometrial stroma and, to a lesser extent, in the base of the lining epithelium (Rodriguez-Martinez et al., 1990; Bischof et al., 1994; Kaeoket et al., 2003; Robertson, 2007), a picture not seen in the oviduct, except for the mesothelial-covered infundibulum (Jiwakanon et al., 2006) or the presence of lymphocyte-like cells in the base of the SR and the adjoining isthmus segment (Rodriguez-Martinez et al., 1990).

What causes this transient PMN influx and the apparent changes in immune cell phenotypes over time is yet unclear, but components of the SP seem to play a role. The SP initiates the transitory inflammatory response (O'Leary et al., 2004) followed by a secondary recruitment of antigen-presenting cells (macrophages and dendritic cells), prerequisite for the generation of paternal antigen-specific maternal T cells (Schuberth et al., 2008) that can recognize and respond to paternal major histocompatibility (MHC) antigens. However, prevention of the development of maternal type-I immunity to, particularly, conceptus antigens is essential for pregnancy, and it is attained by induction of a transient state of peripheral immune tolerance by the female (Robertson et al., 2009).

SP proteins are a major component of the boar ejaculate (39.4 ± 13.45 mg/mL, Rodriguez-Martinez et al., 2005), of which 80–90% are of vesicular gland origin, with 75–90% of them belonging to the spermadhesin family; the alanine-glutamine-asparagine proteins AQN (-1 and -3), the alanine-tryptophan-asparagine proteins [AWNs] and the porcine seminal plasma proteins I and II [PSP-I and PSP-II] (reviewed by Calvete and Sanz, 2007). Spermadhesins are multifunctional 12–16 kDa glycoproteins that attach to the sperm plasma membrane to various degrees from the testis to the ejaculate and whose biological activities depend on their sequence, grade of glycosylation or aggregation state, as well as their ability to bind heparin. The AQN-1, AQN-3 and AWN are grouped as heparin-binding proteins or HBPs (Calvete et al., 2005). Spermadhesins are involved in sperm membrane stabilization, capacitation, and sperm-oviduct and zona pellucida (ZP) interplay (Caballero et al., 2008). Moreover, PSP-I and PSP-II, which account for >50% of all SP proteins (Garcia et al., 2008) can bind to pig lymphocytes (Yang et al., 1998), enhance their proliferation (Leshin et al., 1998) and act as leukocyte chemoattractant in rodents (Assreuy et al., 2002, 2003). It remains to be determined whether specific boar SP proteins act also in the female pig *in vivo*.

The aim of the present study was, therefore, to test whether pig HBPs and PSPs, isolated from the SP of SRF samples collected from mature, fertile boars could recruit different lymphocyte subsets and PMNs into the superficial endometrium, respectively, the lining epithelium and the lumen of the pig uterus *in vivo*, and thus be responsible for triggering changes in the female in response to the entry of spermatozoa at mating. Selected results of these experiments have previously been presented elsewhere (Rodriguez-Martinez et al., 2009)

1.2 Material and Methods

1.2.1 Animals

Mature boars (Swedish Yorkshire and Swedish Landrace, $n = 5$) 2–5 years old, kept in individual pens with females in the close neighbourhood and selected for normal semen quality and proven fertility, were used as semen providers for SP collection. Crossbred sows ($n = 14$) with normal reproductive performance prior to the study, weaned after a lactation of 3 or 5 weeks (weighing between 160 and 230 kg at weaning) and a mean parity of 4 farrowings (range of 2–6) were used. The sows were, following clinical examination on site, either kept at a commercial farm having common research facilities with the Department of Medicine and Animal Surgery, University of Murcia, Spain ($n = 6$), or purchased from a

Swedish commercial herd and brought directly after weaning to the Division of Reproduction, Swedish University of Agriculture Science (SLU), Uppsala, Sweden (n = 8). Sows were either relocated into individual crates present in a mechanically ventilated confinement facility (Spain) and fed once a day with a commercial diet (2.5 Kg/d, 15% protein, 3% fat, 6% cellulose, 6.35% ash and 0.75% lysine), or individually housed in straw-bedded pens with mechanical ventilation and fed according to Swedish standard (Simonsson, 1994) for dry sows (barley-based sow diet, 14.5% protein and 12.5 MJ/kg of metabolizable energy), 2.5 kg/day. Water was provided *ad libitum*. Beginning on the day after weaning, all sows were checked for detection of oestrus twice a day (0700 and 1900 h) by experienced operators, in the presence of a boar. The experimental protocol had previously been reviewed and approved by the Bioethical Committee of the University of Murcia, Murcia, Spain (Exp. 1) or the Local Ethical Committee for Experimentation with Animals, Uppsala, Sweden (Exp. 2).

1.2.2 Isolation of SP spermadhesins

To obtain the HBPs and the PSP-I/PSP-II, sperm-rich ejaculate fractions (SRF, 100 mL) were collected by the gloved-hand method. The SP was separated from spermatozoa by centrifugation at $800 \times g$ for 15 min at 20°C, and the supernatants were sequentially filtered through 10 μm and 1.2 μm filters and pooled. The HBPs and the PSP-I/PSP-II were isolated as described by Calvete et al. (1995). The identity and purity of the protein preparations were assessed by automated Edman degradation (using an Applied Biosystems Procise N-terminal sequencer) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (using sinapic acid saturated in 0.1% trifluoroacetic acid and 70% acetonitrile as matrix and an Applied Biosystems Voyager-DE Pro instrument operated in delayed extraction and linear modes). Amino acid analysis (after hydrolysis in 6N HCl at 110°C for 24 h in evacuated and sealed ampoules) was used to quantify the amount of either protein, which averaged ~15 mg/mL.

1.2.3 Experiment 1: Determination of lymphocyte subsets (CD2, CD4 and CD8) and PMNs recruited into the porcine superficial endometrium in vivo, following infusion of HBPs or PSP-I/PSP-II to anaesthetized oestrous sows

The oestrous sows (n = 6) were sedated by azaperone i.m. administration (2 mg/Kg b.w., Azaperone, Abbott, Spain) before being induced to general anaesthesia by an i.v. injection of thiopental sodium (7 mg/kg b.w., Pentotal, Abbott, Spain) and maintained by narcosis with

inhalatory isofluorane (3.5–5%, Isoflurane, Hoeschst, Germany). A midline incision was done to expose the reproductive tract and to reach (one side at a time) the uterine horn tip and the ipsilateral oviduct and ovary, to confirm pre-ovulatory status. Long plastic catheters were passed through the cervix into each of the uterine horns for infusion, and one of the uterine horns was randomly clamped close to the uterine body to provide two separate infusion horn units. One of the uterine horns was randomly used as intra/animal control, infused with 100 mL of saline, to provide baseline cell presence, while the contralateral horn was randomly infused with either HBPs ($n = 3$) or PSP-I/PSP-II heterodimer ($n = 3$), at 3 mg/mL dose (in 100 mL of saline). Samples ($\sim 5 \times 5 \times 5$ mm) of the superficial antimesometrial endometrium were surgically collected for biopsy at 10–15 cm distance from each other, albeit at random location, at various intervals (2, 10, 30, 60 and 120 min after infusion), fixed in 2% paraformaldehyde solution in 0.067 M sodium cacodylate buffer (pH 7.2, 500 mOsm) and conventionally embedded in paraffin wax. Sections were mounted on polylysine-coated slide glasses and subjected to an antigen-retrieval method (microwave heat-induced antigen retrieval, Shi et al., 1991), in order to unmask epitopes usually masked during the fixation and paraffin-embedding process, prior to immunohistochemistry (IHC). Complementary sections were stained with haematoxylin and eosin (HE) for histological screening.

The IHC was used to characterize some leukocyte subpopulations according to their expression of cluster cell surface antigens (Piriou-Guzylack and Salmon, 2008), by means of mouse monoclonal antibodies (mAbs) towards CD2, CD4 and CD8 (VMRD, Pullman, WA, USA). Tris-buffered saline (TBS), 0.05 M pH 7.6 was used for all dilutions and washings. Single IHC-labellings were performed using a standard avidin-biotin immunoperoxidase technique (Vectastain ABC kits, Vector Laboratories, Burlingame, CA, USA). The deparaffinized, microwave-treated sections were pre-incubated with 10% normal goat serum (NGS, 2×30 min) to diminish non-specific protein binding, within incubating humid chambers. The sections were then overlaid with 50 μ L of the primary mAbs and incubated at +4°C, overnight, followed by rinsing and 30 min incubation with the secondary biotinylated antibody (goat anti-mouse, 1:1000, Vector Laboratories, Burlingame, CA, USA). Use of normal mouse IgG (Santa Cruz, CA, USA) and omission of the primary mAbs on complementary endometrial sections constituted negative controls, while lymph node sections were used as positive controls. Diaminobenzidine (DAB, Dakopatts, Älvsjö, Sweden) was used as chromogen to visualize immunostained cells. Following fixed-time counterstaining

with Mayer's haematoxylin (H) and mounting in glycerine gelatin, sections were examined with bright-field light microscopy.

Counting of IHC-marked T cells (H-stained sections) and of PMNs (HE-stained sections) in coded tissue samples for biopsy was done with a Nikon FXA photomicroscope (Nikon, Tokyo, Japan) at $\times 400$ using an ocular reticle (tissue area: 0.0625 mm^2) divided in small squares, placed in one ocular eyepiece, by one and the same operator. Particular attention was given to the lining epithelium and the subjacent lamina propria, avoiding preparation artefacts. Counting was carried out along the length of the surface epithelium and the entire area of the lamina propria contained within the reticulum, on between 7 and 10 ocular reticle fields. The relative number of cells was quantified in treatment tissues against control tissues, at each time interval.

1.2.4 Experiment 2: Degree of recruitment of PMNs in the uterine lumen following a single cervical insemination of PSP-I/PSP-II heterodimer in conscious oestrus sows

Eight multiparous (parity 2–4) crossbred sows were, following detection of standing oestrus, randomly allotted to one of two equal groups, control or treatment. The control group was infused intra-utero (mimicking cervical AI *ad modum*) with sterile physiological saline solution (100 mL dose, 0.9% NaCl), whereas the treatment group was infused with PSP-I/PSP-II heterodimer in physiological saline solution (3 mg/mL, 100 mL dose). All sows were inseminated ~ 12 h after onset of behavioural oestrus. The sows were slaughtered 3 h after infusion, and the genitalia promptly removed and macroscopically examined for normality. The uterine horns were divided into right and left by surgical clamps, leaving 5 cm segments (one ad-tubal and another ad-uterine) for histological examination, which were immersion-fixed with a 2.5% solution of glutaraldehyde in cacodylate buffer. The remaining mid-segments (~ 1 m long) were flushed with 50 mL of saline solution, and the flushing was collected in 50 mL conical centrifuge tubes (BD Falcon, San Jose, CA, USA). Pressure on the tissues was consistently avoided to minimize blood contamination of the flushing and, to ensure the samplings per animal were uniform, collection was stopped, once 50 mL of uterine fluid contents per uterine horn was collected. The 50 mL flushed fluid was centrifuged ($300 \times g$), the upper 85 mL discarded, and the remnant 15 mL containing a pellet was examined for cell content. Cells were counted with a Bürker haemocytometer and

distinguished as leukocytes and other cells (erythrocytes , epithelial cells) in Papanicolau-stained smears. Cell numbers were expressed as PMNs per mL.

The fixed tissue specimens were post-fixed in 2% OsO₄, dehydrated in increasing ethanol concentrations and infiltrated with Agar 100 (Agar Scientific, Stansted, Essex, UK) plastic resin for transmission electron microscopy (TEM). The blocks were cut on an ultramicrotome (Reichert, Vienna, Austria). Semi-thin sections (1 µm thick) were routinely stained with toluidine blue, and areas representative of each section were selected for TEM, from which ultrathin sections (~60 nm thick) were cut and picked up on copper grids, counter-stained with uranyl acetate and lead citrate and examined in a Philips EM 420 TEM microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 80 kV.

1.2.5 Statistical analysis

All results are expressed as mean ± SD. Cell countings were log-transformed before analysis. Statistical evaluation was undertaken using the SAS statistical package (Statistics Analysis Systems Package (SAS Institute, Cary, NC, USA, version 9) by analysis of variance (ANOVA, Proc Mixed). The statistical model included the effects of uterine side (Exp. 1)/group (Exp 2.) and sampling interval, their interaction and the random effect of sow nested within group (Exp. 2). Student *t*-test was used for pair-wise comparisons between control and treatment at each time (Exp. 1) or group (Exp. 2) when an overall significance was found. A $P < 0.05$ was considered statistically significant.

1.3 Results

1.3.1 Experiment 1

The data, depicted in **Figure 1** (T cells) and **Figure 2** (PMNs), respectively, show the intensity of recruitment, based on the relative number of cells counted in the treatment tissues taken for biopsy, related to those cell numbers recorded in the control tissues (baseline 0) at each time interval (2–120 min). Regarding the IHC, very few cells (3.8 ± 0.5) positive (+) for CD2, CD4 nor CD8 were seen in the epithelium or the lamina propria within a unit field (0.0625 mm^2) immediately after saline infusion in the control uterine horn, and they did not significantly increase over time ($P > 0.05$). Infusion of spermadhesins gave different responses (**Figure 1A–C**). Infusion of PSP-I/PSP-II induced a significant 4- to 7-fold increase in CD2⁺ relative cell numbers from 10 min onwards ($P < 0.01$), which was not followed by

CD4⁺ cells ($P > 0.05$). CD8⁺ relative cell numbers increased significantly (3-fold, $P < 0.05$) after 60 min of the infusion of PSP-I/PSP-II. Infusion of HBPs, on the other hand, did not cause any increase in CD2⁺ cells, until 120 min of the infusion, where a 1-fold increase was registered but with large variation among sows ($P > 0.05$). A late, significant ($P < 0.05$) 3-fold increase was seen in CD4⁺ cell numbers, however. HBP-infusion showed a sustained 1–2-fold increased level of CD8⁺ cells over time, albeit nonsignificant ($P > 0.05$).

Compared to control side (saline-infused uteri), exposure to the PSP-I/PSP-II heterodimer significantly ($P < 0.05$) induced the migration of PMNs to the surface uterine epithelium, after just 10 min of infusion (**Figure 2**), a recruitment that was sustained over the experimental period, becoming 5-fold by 30 min and 7-fold higher from 60 min onwards ($P < 0.001$). PMNs were detected in the lumen of tissue samples collected at 30 min and thereafter. The infusion of a similar dose of HBPs had no significant effect ($P > 0.05$).

1.3.2 Experiment 2

The majority of the cells encountered in the flushings were leukocytes ($77.3 \pm 24.6\%$), followed by erythrocytes ($20.2 \pm 15.3\%$) and epithelial cells ($5.8 \pm 4.2\%$, mean \pm SD), with significant differences for the proportions of leukocytes between treatment and control groups ($96.1 \pm 3.35\%$ vs. $57.4 \pm 24.7\%$ respectively, $P < 0.05$). No significant differences were seen between sides within each sow ($P > 0.05$), but individual differences were noted within treatment. Among leukocytes, >96% of them were morphological PMNs, while $3.41 \pm 1.23\%$ were lymphocyte-like cells. As seen in **Figure 3**, the simple infusion of saline (control) was able to elicit migration of PMN into the uterine lumen (5.8 ± 4.62 million PMN/mL, mean \pm SD). However, a 6-fold higher mean number of PMN/mL (35.4 ± 12.56 million PMN/mL, mean \pm SD, $P < 0.001$) was recovered from the uterine lumen of sows inseminated with the PSP-I/PSP-II heterodimer ($P < 0.001$), thus confirming that, at doses 5-fold lower than those present in the boar ejaculate, the heterodimer induced *in vivo* PMN migration in the pig. **Figure 4** depicts a representative entry of PMNs 3 h after the AI infusion of PSP-I/PSP-II.

1.4 Discussion

The results of the present study clearly show that the PSP-I/PSP-II heterodimer (but not HBPs) induced a time-dependent influx of PMNs into the uterine lumen of anaesthetized and

conscious oestrous sows, at doses 5 times lower than those often present in the SP of boars. These effects confirm the results of Assreuy et al. (2002), in which infusion of porcine PSP-I/PSP-II into the peritoneal cavity of rats caused a dose-dependent, time-responsive migration of PMN and monocyte leukocytes, typical of inflammation. The overall results suggest the PSP-I/PSP-II spermadhesin acts as a post-mating inflammation mediator in pigs.

In the pig, as in most mammals studied so far, deposition of semen (via natural mating or by AI of neat or extended semen) into the uterine cavity elicits a massive invasion of PMNs towards the lumen, followed by NETs formation and sperm phagocytosis. These PMNs are originally present in the lamina propria in control, unmated oestrous gilts, their extravasation considered related to the high pre-ovulatory oestrous levels of oestrogens in pigs, since chemokine expression and increments in T-cell numbers are considered oestrogen-induced (Robertson et al., 2009). However, we still need to confirm this as the reason or determine other reasons for this PMN accumulation. In the same study by Rodriguez-Martinez et al. (1990), where gilts were inseminated with fresh neat semen 12 h after onset of oestrus, the PMNs invading the lining epithelium became associated with intra-epithelial macrophages, entered the uterine lumen and actively phagocytosed spermatozoa, during a window period of 3–6 h. Interestingly, such PMN-macrophage interaction was not seen in the oviductal sperm reservoir, where spermatozoa had colonized the segment and were conspicuously intact (Rodriguez-Martinez et al., 1990). On the other hand, lymphocyte-like cells were present in the sperm reservoir (SR).

The rationale for the above-mentioned PMN intra-uterine invasion has been already discussed; it acts as a primary inflammatory reaction to cleanse the intra-uterine lumen from foreign cells, proteins and eventual pathogens, so that the environment prepares for the descending 4-cell embryos. The inflammatory reaction induced by porcine PSP-I/PSP-II in rats (Assreuy et al., 2002) showed a PMN peak 4 h after, which corresponds to findings in pigs (Rodriguez-Martinez et al., 1990) and in cows (Alghamdi et al., 2009) and, partly, with the present results, where a clear trend of sustained entry was registered in the tissues collected for biopsy from 60 min onwards. Peak-like PMN numbers would most likely correspond to those accounted for in Exp. 2, where sows were euthanized by ~3 h after infusion of PSP-I/PSP-II. The PMN numbers, large as they were, were higher than those reported by O'Leary et al. (2004), using gilts, but lower than those accounted for by Woelders and Matthijs (2001) and Matthijs et al. (2003), most likely due to methodological differences,

since these latter authors counted PMNs and spermatozoa in homogenized tissues, thus accounting for both luminal PMNs (as we did) and those contained in the uterus diverse tissues, while we restricted the volume collected to a fixed volume of saline flushing post-mortem. In any case, our current results were distinct; infusion of PSP-I/PSP-II induced a marked entry of PMNs to the uterine lumen. Although there was also a restricted entry after infusion of the common saline vehicle, such effect ought to be caused by the volume-elicited distension of the uterine lumen, as shown by Woelders and Matthijs (2001).

Our current results (Exp. 1) also showed the invasion of PMNs did not immediately reach the uterine lumen, since PMNs were first seen there by 30 min post-infusion, findings that confirm previous studies (Lovell and Getty, 1968; Bischof et al., 1994). Such lag allows a window of opportunity for a certain subpopulation of ejaculated spermatozoa to traverse the uterine lumen without risking phagocytosis. Spermatozoa deposited by natural mating or AI in oestrous pigs reach the oviducts just a few minutes after (Hunter 1981), and basically, they should not encounter PMNs along their transit. Once they enter the sperm reservoir in the oviduct, they face a safe period prior to eventual migration to the site of fertilization (Rodriguez-Martinez, 2007). Interestingly, our own experimental *in vivo* studies have shown that those pig spermatozoa ejaculated in the sperm-peak portion of the SRF (the 1st 10 mL of the SRF, the so-called P1; see Rodriguez-Martinez et al., 2009, for a review) appear overrepresented in the tubal sperm reservoir by 3 h post-AI (Wallgren et al., 2009). This suggests that the primary transport of spermatozoa through the uterus is rather quick, allowing some P1 spermatozoa to safely reach the reservoir (which is, thereafter, replenished with other spermatozoa than those in the P1). Overall, the findings suggest the function of the PSP-I/PSP-II might primarily be related to its pro-inflammatory inductive role. Whether this effect is coupled to the relative concentration of PSP-I/PSP-II is yet to be disclosed. The pig is one of the species containing a large amount of SP spermadhesins (~35–45 mg/mL), of which at least ~20 mg/mL would most likely be PSP-I/PSP-II (Calvete and Sanz, 2007). Spermadhesins are present throughout the fractions of the ejaculate, derived from testis or epididymides (first portion of the SRF) and from the vesicular glands (i.e. from the SRF onwards). The concentration of spermadhesins increases alongside the increasing secretion of the vesicular glands, while the sperm-peak portion of the ejaculate (the P1) contains ~1–1.5 mg/mL of PSP-I/PSP-II, for example, ~20 times less than the relative concentration in the bulk ejaculate (see Rodriguez-Martinez et al., 2009). Whether this means that a relationship exists between exposure to lower PSP-I/PSP-II amounts and sperm viability of potentially

fertilizing spermatozoa (at least *in vivo*) remains to be proven. Interestingly, highly extended boar spermatozoa maintain sperm viability and fertilizing capacity *in vitro* when exposed to similar amounts of PSP-I/PSP-II as those found in the P1 (Caballero et al., 2004a–b, 2005, 2006), by mechanisms not yet fully understood. Also interestingly, HBPs, which bind to the boar sperm membrane, remain on the rostral membrane until capacitation *in vivo* (Calvete et al., 1997), or on the post-equatorial membrane until reaching the zona pellucida *in vivo* (Rodriguez-Martinez et al., 1998) or *in vitro* (Dapino et al., 2009), but usually fail to preserve sperm viability of highly extended boar spermatozoa *in vitro* (Centurion et al., 2003).

Obviously, there is a need for further studies to discern whether this is a matter of the concentration of glycoproteins or their type of attachment to the spermatozoa. In any case, HBPs (at the same dosage as PSP-I/PSP-II) were clearly unable to elicit an invasion of PMNs to the luminal epithelium, as clearly shown in the present study, even considering there were few animals studied. Moreover, we tested only a single dose and only for a period of 120 min, which impairs further comments and calls for further experiments. To prolong the sampling would imply a longer narcosis period, which would have welfare concerns. Likewise, use of several protein doses would require a rather large number of specimens. Studies could be done *in vitro*, of course, but this would lead to the paradoxical results of SP experiments present in the literature (Veselsky et al., 1991; Bischof et al., 1994; Rozeboom et al., 2001a–b; O’Leary et al., 2004, 2006; Taylor et al., 2008, 2009b; Alghamdi et al., 2009), regarding the pro-inflammatory respectively the attenuating effects of the SP.

For how long does the inflammation persist? Few studies have been done to determine the exact duration of the inflammation itself, and they focused on the presence of invading PMNs (Lovell and Getty, 1968; Rodriguez-Martinez et al., 1990; Bischof et al., 1994; Alghamdi et al., 2009). They mostly agree that the PMN-peak is reached somewhere between 3 and 6 h after semen deposition. Such interval agrees with the findings by Assreuy et al. (2002), using porcine PSP-I/PSP-II in a rodent model, where the PMN invasion to the site of exposure peaked at 4 h. Inflammation does not stop there, since it has many other components, and PMN presence can still be registered 24 h later (Kaeoket et al., 2003). Moreover, there is major individual variation among females in coping with the inflammatory response, and it is well established that some pig females develop long-lasting post-mating inflammation that evolves into endometritis (De Winter et al., 1992). In any case, intervals between conventional AIs are longer than 3–6 h, as praxis in pig breeding, thus allowing the PMN

massive presence to resume before a new inseminate is placed. A matter for further discussion is whether the inflammatory response is elicited also when extended, liquid preserved boar semen or, for that matter, even frozen-thawed semen are inseminated. The answer is at first sight, yes, but the reasons differ. For production of liquid AI semen doses, the ejaculate is extended with protein-free extenders, and the SP is simply diluted, but not eliminated. Depending on the type of ejaculate collected (whole ejaculate or only the SRF), the amount of spermadhesins differs, but it is most likely kept at levels around or above 3 mg/mL, thus being able to elicit a response similar to the one shown in the current study. Semen collected for freezing comprises, most often, the SRF only, where the amounts of spermadhesins are not highest. Moreover, the semen is often extended and centrifuged to remove the major part of the SP. However, despite this removal, the frozen-thawed semen is able to cause inflammation post-AI, but the cause is still disputed, since some extenders include proteins that are pro-inflammatory (Taylor et al., 2009a–b). In conclusion, the PSP-I/PSP-II at relatively low concentrations is a post-mating (or post-AI) inflammatory mediator in pigs.

However, there are other mechanisms that induce PMN migration to the surface epithelium and lumen, via pro-inflammatory soluble cytokines (such as IL-1 or IL-8, Assreuy et al., 2003; Sharkey et al., 2007; Scott et al., 2009) of macrophage or mast cell origin (Ribeiro et al., 1991). These cells are common in the porcine uterus (Kaeoket et al., 2003) and invading PMNs have been seen to interact with intra-epithelial macrophages (Rodriguez-Martinez et al., 1990). Mast cells are also able to produce IL-4 and IL-10, inhibitory of inflammation (Ribeiro et al., 1991). The picture is much more intricate. While PSP-I has been shown to act directly on PMN activation, PSP-II activates PMNs indirectly, most likely via macrophages (Assreuy et al., 2002). We have used the heterodimer, thus calling for further studies.

Induction of PMN invasion, as a token for inflammation, is not the only effect of the SP on the female. The SP also mitigates the immune responses by the female to paternal antigen-bearing spermatozoa or early embryos in the oviduct (immuno-privileged area) or in the uterus (developing embryos/foetuses and their placentae), by eliciting a transient state of peripheral immune tolerance (O'Leary et al., 2004; Robertson et al., 2009). Such tolerance is apparently partly mediated by regulatory T (Treg) cells, a conspicuous sub-population (5–10% in rodents) of CD4⁺ T cells, which are identifiable because they constitutively express the interleukin receptor CD25⁺ and the transcription factor FOXP3 (Zenclussen, 2006). Treg cells strongly suppress the generation and effector function of Type I (cell-mediated) immune

responses, provided they are activated by antigens, presented by dendritic cells, and proliferate (Zenclussen 2006). Very recently, Robertson et al. (2009) clearly demonstrated in mice that components of the SP originated in the seminal vesicles induced an expansion of the Treg cell pool, which, by inducing a transient state of peripheral immune tolerance, suppressed the type-I immune response to male alloantigens. Considering that the conceptus is, until eclosion and expansion, covered by the zona pellucida, it appears that the generation of paternal antigen-specific tolerance in early pregnancy must be initiated at mating or AI by the exposure to semen, and apparently, particularly to the SP (Robertson et al., 2009), causing expression of lymphocyte activation markers and of cytokines (Johansson et al., 2004). Several of the same antigens expressed from paternal genes by the conceptus are actually present in semen, including MHC antigens and minor antigens. Semen deposition is associated with the expression of lymphocyte activation markers and cytokines, apparently modulated by factors present in the SP. Seminal plasma upregulates MHC class II and interleukin-2 (IL-2) receptor expression (Bischof et al., 1994) and stimulates production of interleukin-6 (IL-6) by the uterine epithelium within 3 h of SP exposure (Madej et al., 2009). As well, SP stimulates expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), and the monocyte attractant protein-1 (MCP-1) (O'Leary et al., 2004). This cascade of induced changes leads to a transition of leukocyte phenotypes, with PMNs being first replaced by monocytes (peaking by 96 h, Assreuy et al., 2002), and later by macrophages and dendritic cells (Robertson, 2007). The female uterus is well equipped with antigen-presenting cells when semen deposition is issued, cells that can take up the antigen, process it and present the antigenic peptides to Treg cells. Semen, the SP in particular, induces—by eliciting a local inflammatory response—the expression of pro-inflammatory cytokines and chemokines in uterine epithelial cells, causing recruitment of macrophages, dendritic cells and granulocytes, as indirectly illustrated previously (Rodriguez-Martinez et al., 1990).

In the current study, the infusion of PSP-I/PSP-II into the uterus of sows *in vivo* significantly recruited (more clearly than that of HBPs at the relatively same dosage), some uterine lymphocyte subsets (Gerner et al., 2009), such as CD2⁺ (T_H, NK, cytokine-releasing cluster) from 10 min onwards and of CD8⁺ (cytolytic) cells from 60 min. These findings suggest that the PSP-I/PSP-II heterodimer might also participate in the initial reshaping of the T-cell component of the immune response towards semen deposition. Infusion of HBPs, on the other hand, showed late increases (some not really significant) in CD2⁺ and CD4⁺ (helper cells) as well as a sustained 1–2-fold increased level of CD8⁺ cells over time. Recently, Moldenhauer

et al. (2009) have shown that murine SP paternal antigens, delivered via female antigen-presenting cells and immune-deviating cytokines, drive the activation and expansion of the paternal antigen-reactive CD4⁺ and CD8⁺ T-cell populations to, ultimately, mediate female tolerance to embryo presence and attainment and maintenance of pregnancy. Whether the findings in the present study represent the only changes occurring or become more definitive later on, could not be determined, since Exp. 1 encompassed only 2 h of exposure period. Since T cells generally take several days to generate a robust response after stimulation, longer periods are needed, but might be difficult to obtain using *in vivo* protocols.

The SP of the boar contains immune-regulatory molecules, including high concentrations of the potent immunosuppressive transforming growth factor- β (TGF- β) multifunctional cytokine group (Robertson et al., 2002), which, mediated by Treg (in rodents), induce differentiation of suppressor T cell phenotypes, to reach a state of adaptive functional immune tolerance to male antigens by the female (O'Leary et al., 2004; Robertson et al., 2006, 2009; Robertson, 2007), most relevant to early pregnancy and, further, to fertility. The SP from different boars varies largely in its contents of cytokines, which in turn leads to differential expression of endogenous cytokines in the females (Robertson, 2007). A differential SP induction of maternal tolerance might thus relate to the often-observed differences in embryo survival among sires (e.g. innate fertility), a real long-lasting effect of the SP on the female.

1.5 Conclusions

In sum, the data provided clearly show that PSP-I/PSP-II heterodimer (but not HBPs at the same relative dosage) induced influx of PMNs into the uterine lumen of anaesthetized and conscious oestrous sows, at doses 5 times lower than those present in the SP of boars. As well, the findings confirmed that there is a period of latency for PMNs' migration to the uterine lumen (<30 min), implying that—considering the presence of different signalling proteins in the various SP fractions—there might be a window of opportunity for a certain subpopulation of ejaculated spermatozoa, bathing in low amounts of PSP-I/PSP-II, to traverse the uterine lumen without risking phagocytosis. Moreover, high concentrations of PSP-I seem to hamper sperm function (Caballero et al., 2008; Saravia et al., 2009) and, interestingly, a recent study by Novak et al. (2009) has marked PSP-I as negatively related to *in vivo* fertility. Infusion of the spermadhesins PSP-I/PSP-II, and also of HBPs, significantly recruited some

uterine lymphocyte subsets, after PSP-I/PSP-II for CD2⁺ from 10 min onwards; after HBPs for CD4⁺ by 120 min; and for CD8⁺ cells from 60 min after PSP-I/PSP-II infusion, *in vivo*. Thus, spermadhesins might also participate in the initial reshaping of the T-cell component of the immune response towards semen deposition, perhaps by providing antigen and cytokine signals to expand populations of relevant T-cells (such as Treg). The overall view is that spermadhesins, by their temporal appearance and amount, are able to modulate fertility of the inseminate and can, therefore, be used as markers for fertility in boars.

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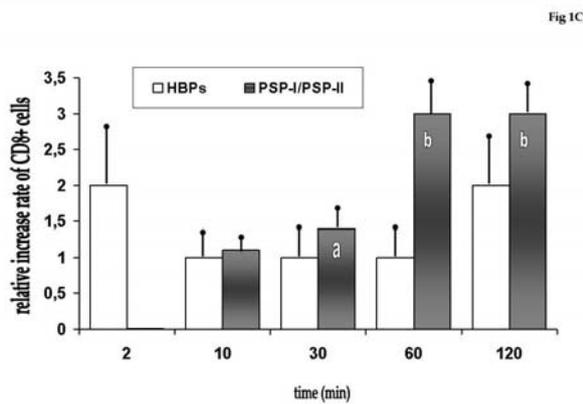
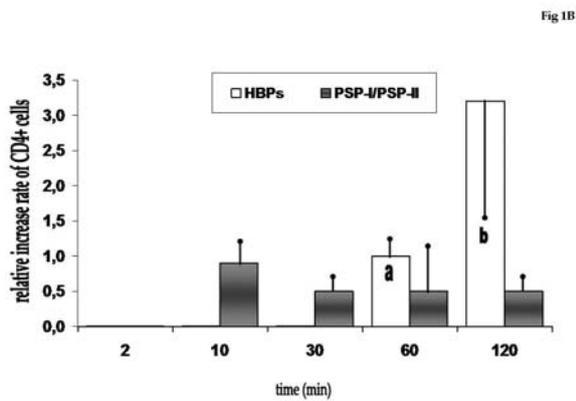
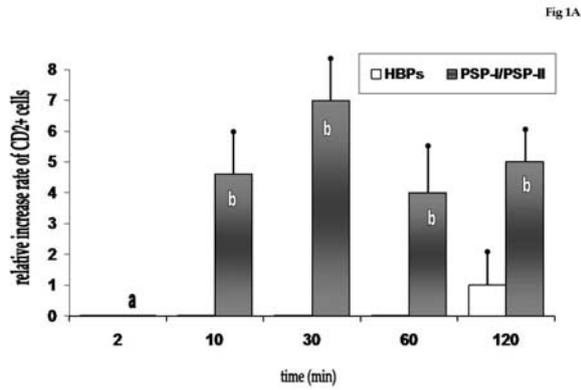


Figure 1A–C. Rate of recruitment (increase rate of the number of immunostained cells within 0.0625 mm^2 of sectioned tissue in treatment horn above those cell numbers in control horn as baseline, means \pm SD) of lymphocyte subsets (A: CD2^+ , B: CD4^+ and C: CD8^+) to the superficial endometrium of oestrous sows ($n = 6$) at various times (2–120 min) after infusion of HBP or PSP-I/PSP-II spermadhesins (3 mg/mL in 100 mL saline). Contralateral uterine horns were infused with 100 mL saline. ^{a-b}mark significant differences between sampling times ($P < 0.05$).

Fig 2

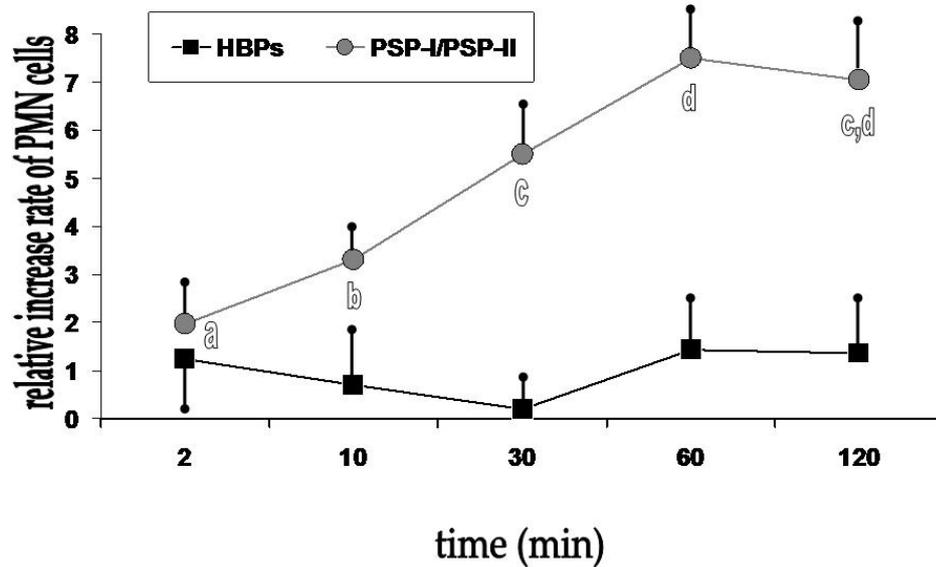


Figure 2. Rate of PMN entry (increase rate in the number of PMNs within 0.0625 mm² of sectioned tissue in treatment horn above those PMN numbers in control horn as baseline, means \pm SD) to the endometrial lining epithelium of oestrous sows (n = 6) at various times (2–120 min) after infusion of HBP or PSP-I/PSP-II spermadhesins (3 mg/mL in 100 mL saline). Contralateral uterine horns were infused with 100 mL saline. ^{a-d}mark significant differences between sampling times ($P < 0.05$).

Fig 3

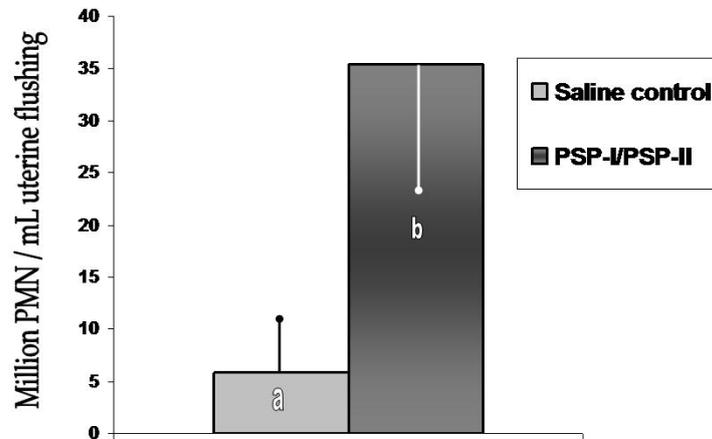


Figure 3. Mean numbers (\pm SD) of PMNs flushed from the uterine lumen of oestrous sows ($n = 8$) 3 h after the insemination of 100 mL of saline solution without (control) or with spermadhesin PSP-I/PSP-II (3 mg/mL). ^{a-b}mark significant differences between sampling times ($P < 0.05$).

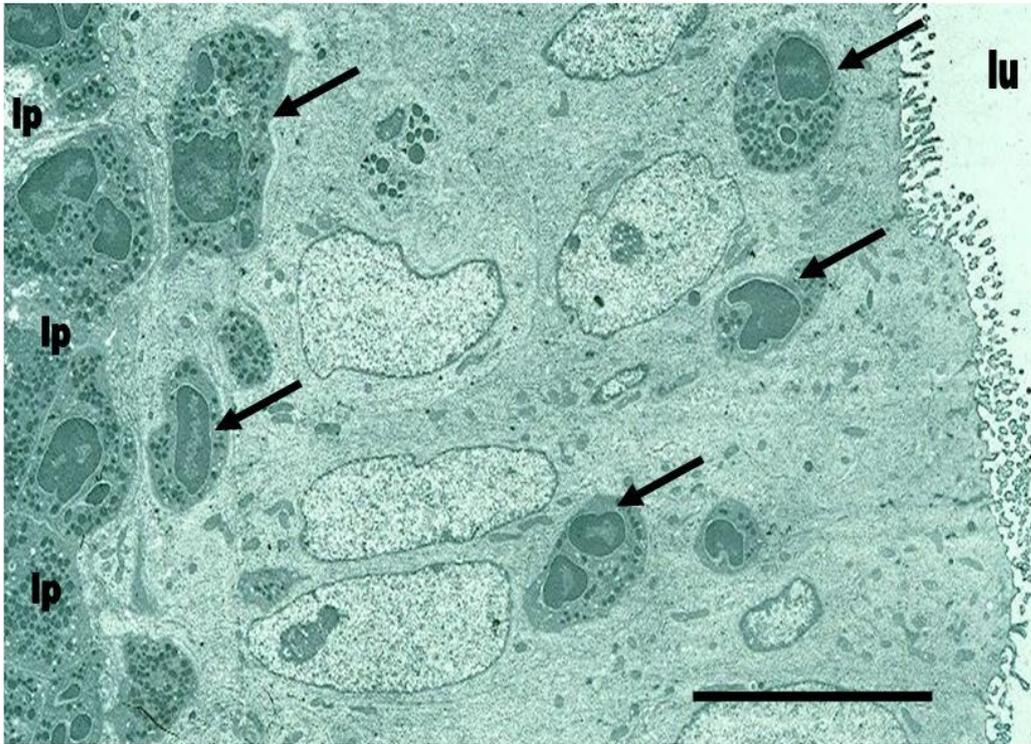


Figure 4. Transmission electron micrograph showing the entry of polymorphonuclear leukocytes (PMNs, arrows) from the lamina propria (lp) into the lining epithelium and towards the lumen (lu) of the uterus in an oestrous sow, 3 h after the infusion of PSP-I/PSP-II (3 mg/mL). Bar: 10 μ m.