Published-Ahead-of-Print June 28, 2006, DOI:10.2164/jandrol.106.000539
Journal of Andrology, Vol. 27, No. 6, November/December 2006

DOI: 10.2164/jandrol.106.000539

Immunolocalization and Possible Functional Role of PSP-I/PSP-II Heterodimer in Highly Extended Boar Spermatozoa

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Received for publication May 8, 2006; accepted for publication June 18, 2006.

Abstract

PSP-I/PSP-II heterodimer is a major protein of boar seminal plasma which is able to preserve, in vitro, the viability, motility, and mitochondrial activity of highly extended boar spermatozoa for at least 5 hours. However, little is known about the binding pattern of the heterodimer to the sperm plasma membrane and its eventual relation with the maintenance of the sperm functionality. The present study investigated the effect of exposing highly extended boar spermatozoa (1 million/mL) to 1.5 mg/mL of PSP-I/PSP-II for 0.5, 5, and 10 hours at 38°C on sperm characteristics and the changes in PSP-I/PSP-II localization as a result of both the addition of PSP-I/PSP-II to the extender and the incubation time. Exposure of the spermatozoa to PSP-I/PSP-II preserved sperm viability, motility, and mitochondrial activity when compared to nonexposed spermatozoa. This protective effect lasted for 10 hours ($P < .05$). After immunolabeling of highly extended semen with rabbit monospecific polyclonal antibody against PSP-I/PSP-II, the percentage of immunopositive spermatozoa declines over time from 71% (0.5 hours) to 49% (10 hours). However, more than 80% of spermatozoa remained labeled during the 10-hour incubation period if PSP-I/PSP-II was added. Scanning electron microscopy revealed 4 different binding patterns. The heterodimer was mainly localized to the acrosomal area, being redistributed to the postacrosomal area or lost during in vitro incubation. In conclusion, the protective effect of the heterodimer appears to be related to its adhesion to the

acrosomal area, and the loss of this protective effect coincides with a stepwise redistribution of PSP-I/PSP-II during incubation.

Key words: Reproductive tract, semen, sperm, seminal plasma, sperm capacitation

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In the boar, the majority of seminal plasma (SP) proteins belong to the spermadhesin family, a group of (glyco)proteins built by a single CUB domain architecture (Romero et al, 1997) and thought to play important roles in individual steps of the fertilization process, such as capacitation and zona pellucida binding (Töpfer-Petersen et al, 1998). The spermadhesin family comprises 5 members: AQN-1, AQN-3, AWN, PSP-I, and PSP-II. The last 2 form a glycosylated PSP-I/PSP-II heterodimer under physiological conditions (Calvete et al, 1995). In vitro, low doses of PSP-I/PSP-II heterodimer (1.5 mg/mL) appear to preserve membrane integrity, motility, and mitochondrial activity of highly extended spermatozoa (Centurión et al, 2003). Processes linked to the removal of factors present in the SP that coat the sperm surface and maintain the stability of the plasma membrane, such as high extension of the spermatozoa (Maxwell and Johnson, 1999), trigger a series of phenomena that resemble those occurring during sperm capacitation, culminating in premature acrosome exocytosis, thus decreasing the life span of the spermatozoa (Maxwell and Johnson, 1999). Although the addition of homologous seminal plasma (from 1% to 10% v/v) is a possible counter-measure to alleviate the consequences of such an "extension effect" (Asworth et al, 1994; Maxwell et al, 1997), differences in SP-protein profiles have been found between males of different fertility. These differences may be related to the variability between different sources of SP (Fournier-Delpech and Thibault, 1993; Killian et al, 1993; Caballero et al, 2004a) which leads to both beneficial and detrimental effects on the spermatozoa.

Either whole SP or specific SP components of low molecular weight affect survivability of boar spermatozoa, depending on how long they are exposed to the PSP-I/PSP-II (Centurión et al, 2003). However, there is an advantage to using an isolated protein instead of whole SP in order to avoid the inherent variability that exists in SP composition between males or ejaculates (Killian et al, 1993; Asworth et al, 1994; Zhu et al, 2000). Previous studies, using various in vitro fertilization treatments, indicated that the PSP-I/PSP-II heterodimer does not sustain its influence for long periods during incubation (Caballero et al, 2004b). This suggests that the heterodimer may either lose its action by blockade or degradation or perhaps by attaching in a loose manner to the plasmalemma, thus losing influence over time. In any case, there is a need to establish the pattern of attachment and the influence of incubation on this pattern, as well as to elucidate whether there is any relationship between the presence of the protein, and its relative concentration, and the protective role in vitro.

The present study examined the relationship between the protective effect of the spermadhesin PSP-I/PSP-II and its binding pattern on the membrane surface of boar spermatozoa subjected to extreme dilution conditions similar to those used for flow cytometric high-speed sorting as performed for chromosomal sex separation.

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Isolation of Spermadhesin PSP-I/PSP-II Heterodimer From Boar Seminal Plasma

All experiments were performed with the SP from sexually mature boars that

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had previously sired offspring. Ejaculates were obtained using the gloved-hand method, and SP was harvested after centrifugation of the sperm suspension at 3800 x *g* at 20 °C for 15 minutes. The supernatants were sequentially filtered through 10- and 1.2-µm-diameter 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 x 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). The identity and purity of the protein preparation was assessed by N-terminal sequence analysis (using an Applied Biosystems 492 automated protein sequencer; Applied Biosystems, Langen, Germany) and MALDI-TOF mass spectrometry using an Applied Biosystems Voyager DE-Pro mass spectrometer. 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}) as determined by Menéndez 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 Analyser (Beckman, Barcelona, Spain). Proteins were dialyzed against distilled water, lyophilised, and stored at −20 °C until used.

Preparation of Anti-PSP-II Antibodies

The PSP-I and PSP-II subunits were purified from the isolated PSP-I/PSP-II heterodimer by reverse-phase HPLC as described by Calvete et al (1995). Polyclonal anti-PSP-II monospecific antibodies were obtained by immunizing female rabbits with subcutaneous inoculations of 0.5 mg of highly purified PSP-II heterodimer in 0.5 mL of PBS emulsified with 1.5 mL of Freund complete adjuvant. The animals were inoculated twice at intervals of 5 weeks after the first immunization with 0.25 mg of the antigen. Two weeks after the last administration, the rabbits were bled through the ear vein and the blood sera were tested for anti PSP-II heterodimer activity by dot-blot ELISA and Western blot.

Semen Handling, Incubation With the Heterodimer PSP-I/PSP-II, and Evaluation of Sperm Parameters

Sperm-rich ejaculate fractions were collected from 3 mature Pietrain boars of proven fertility using the gloved-hand method. Shortly after collection, the semen was extended in Beltsville Thawing Solution (BTS; [Pursel and Johnson, 1975]) to 30 x 10^6 spermatozoa/mL, and the spermatozoa were evaluated for motility. Only ejaculates showing >80% progressive sperm motility were used. The extended ejaculates were pooled and centrifuged (1700 x *g* for 3 minutes), the supernatant discarded, and the final sperm pellet re-extended in PBS to a final concentration of 1 x 10^6 spermatozoa/mL.

One million spermatozoa, extended as described above, were preincubated without or with 1.5 mg/mL of PSP-I/PSP-II (from the same batch of lyophilized proteins) at 38 °C. After 0.5, 5, or 10 hours of preincubation, the sperm plasma membrane, acrosome integrity, mitochondria membrane potential, motility, and binding patterns of PSP-I/PSP-II were evaluated.

Plasma membrane and acrosome integrity were simultaneously assessed by flow cytometry, using the staining protocol described by Nagy et al (2003). For flow cytometric analysis, 50 nM of SYBR-14 working solution (100 µM stock solution in DMSO; component A of LIVE/DEAD Sperm Viability Kit; Molecular Probes Europe, Leiden, The Netherlands), 0.5 µg/mL of PE-PNA (PhycoErythrin-conjugated PeaNut Agglutinin, Biomeda Corp. Foster City, Calif) solution (1 mg/mL stock solution), and 7.5 µM of PI solution (propidium iodide, 1.5 mM stock solution in PBS) were added to 500 µL of sperm suspension. The samples were incubated at 37 °C in the dark for 10 minutes. 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 EXPO 2000 software (Coulter Corporation). Forward and side 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. The SYBR-14 fluorescence was measured through a 525-nm band pass filter, PI fluorescence was collected through a 635-nm band pass filter, and PE-PNA fluorescence was detected through a 575-nm band pass filter. Membrane intact spermatozoa with intact acrosomes were defined as those stained only with SYBR-14.

The JC-1 probe (5,5',6'6-tetrachloro-1,1',3,3'tetraethylbenzymidazolcarbocianyne iodide; Molecular Probes Europe) was used to assess mitochondrial membrane potential of spermatozoa as described by Martinez-Pastor et al (2004), with slight modifications. Briefly, 0.2 µM of JC-1 working solution (3.8 mM stock solution) was added to 500 µL of sperm suspension. Then, samples were incubated at 37 °C in darkness for 10 minutes and analyzed immediately on the flow cytometer (see above). Fluorescence emission of JC-1 monomers and JC-1 aggregates were detected, respectively, in FL1 and FL2 using 520-nm or 590-nm band pass filters.

Sperm motility was estimated with a computer-assisted motility analysis system. Samples (5 µL containing 1 x 10^6/mL 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 microscope (Kanagawa, Japan) equipped with a 10x contrast phase objective and a monochrome video camera (Hitachi CCD, Tokyo, Japan) connected to a personal computer. The sperm analysis was performed using the Sperm Class Analyzer software (Microptic, Barcelona, Spain). The program settings were as follows: frame rate, 25 HZ; search radius, 11.5 µm; minimum track points, 7 frames; and threshold straightness, 75%. Two replicates per sample were examined with at least 100 spermatozoa recorded per replicate for each parameter evaluated. Motion parameters were: percentage of motile spermatozoa, curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and amplitude of lateral head displacement (ALH, µm).

PSP-I/PSP-II Heterodimer Immunocytochemistry

Immunolocalization of the PSP-I/PSP-II heterodimer was studied by both light and scanning electron microscopy (SEM) levels. Sperm suspensions preincubated either without or with PSP-I/PSP-II as described above were smeared onto polylysine-coated glass slides and air-dried for 24 hours. Smears were fixed in 1% paraformaldehyde in PBS for 10 minutes, rinsed in PBS, and incubated with a rabbit monospecific polyclonal antibody against PSP-II (1:500 in PBS) for 120 minutes at room temperature. Samples were rinsed several times in PBS and incubated with Auroprobe EM GAR G10 (10 nm gold labeled goat anti-rabbit IgG; Amersham Biosciences, Uppsala, Sweden) for 100 minutes. After washing in PBS and distilled water, a silver enhancement kit (RPN 491; Amersham) was applied for 10 minutes. Finally, the smears were washed in distilled water and air-dried. To provide positive controls, samples of epididymal spermatozoa were smeared, air-dried, and fixed as above, incubated with the PSP-I/PSP-II heterodimer for 30 minutes, and subjected to the same protocol as above. Negative controls were obtained by omission of the primary antibody.

For evaluation by light microscopy level, incubated samples were photographed with a Nikon Microphot-FXA light microscope (Chibe, Japan) with at least 100 spermatozoa being evaluated per sample at 400x magnification. For evaluation by SEM level (6000x magnification), portions of the glass slides with labeled spermatozoa were cut out, mounted onto metal chucks, critical-point coated with gold-palladium for 15–30 seconds, and examined using a JEOL JSM-6320F SEM (JEOL, Japan), operated at 5 kV.

Statistical Analysis

All data editing and statistical analyses were performed in SPSS version 11.5 (SPSS Inc, Chicago, Ill). Data were subjected to arcsine transformation and analysed by ANOVA using the MIXED procedure according to a statistical model including the fixed effects of heterodimer presence and the random effect of replicate. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered significant at *P* less than .05.

Results

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Figure 1A through C shows the protective effect of PSP-I/PSP-II heterodimer on the spermatozoa exposed to high extension. Addition of the PSP-I/PSP-II heterodimer to a final concentration of 1.5 mg/mL resulted in a greater (*P* < .05) percentage of membrane intact cells at each incubation time period compared with controls (>81% of membrane intact spermatozoa at 10 hours incubation) (Figure 1A). In the absence of PSP-I/PSP-II heterodimer, only 60% of highly extended spermatozoa were viable after 0.5 hours of incubation at 38 °C, and this value significantly decreased to 43% at 10 hours (*P* < .05).

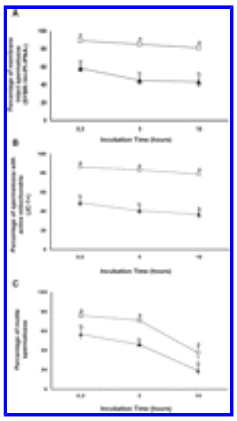

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Figure 1. Effect of the PSP-I/PSP-II heterodimer (□) (1.5 mg/mL in phosphate-buffered saline) on the percentage of membrane intact spermatozoa with intact acrosome **(A)**, mitochondrial activity **(B)** and progressive motility **(C)** of ejaculated boar spermatozoa diluted to a concentration of 1 x 10^6 spermatozoa/mL after 0.5, 5, and 10 hours of incubation at 38°C. Controls (▲) were sourced from the same spermatozoa but incubated in the absence of PSP-I/PSP-II heterodimer. Data are the mean ± SEM of 3 independent experiments. Different letters indicate significantly different values (*P* < .05).

The same trend was observed regarding the mitochondrial activity of highly extended spermatozoa (Figure 1B). After 10 hours of incubation at 38 °C, 1.5 mg/mL of PSP-I/PSP-II preserved the mitochondrial activity in up to 79% of spermatozoa compared with only 37% in controls (*P* < .05).

Sperm motility was better maintained in presence of the PSP-I/PSP-II heterodimer than in controls (*P* < .05), even considering a significant (*P* < .05) decrease of motility being observed after 10 hours of incubation (Figure 1C).

The displacement speed of the incubated spermatozoa (as VCL or VSL) as well as the ALH showed changes over time and following the addition of PSP-I/PSP-II to the incubation medium (Figure 2A through C). The addition of PSP-I/PSP-II heterodimer reduced all 3 variables after 0.5 hours of incubation when compared to controls (*P* < .05). However, from 5 hours of incubation onwards, the variables equilibrated to levels similar to controls.

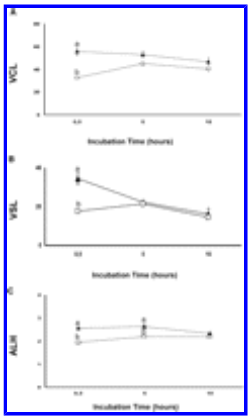

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Figure 2. Effect of the PSP-I/PSP-II heterodimer (□) (1.5 mg/mL in phosphate-buffered saline) on the curvilinear velocity (VCL, µm/s) **(A)**, straight-line velocity (VSL, µm/s) **(B)**, and amplitude of lateral head displacement (ALH, µm) **(C)** of ejaculated boar spermatozoa diluted to a concentration of 1 x 10^6 spermatozoa/mL after 0.5, 5, and 10 hours of incubation at 38°C. Controls (▲) were sourced from the same spermatozoa but incubated in the absence of PSP-I/PSP-II heterodimer. Data are the mean ± SEM of 3 independent experiments. Different letters indicate significantly different values ($P < .05$).

Regarding the immunolocalization of PSP-I/PSP-II heterodimer on the surface of the highly extended spermatozoa, 2 populations could be discerned by light microscope. These were visible either with or without silver aggregates on the sperm head, independently of the addition of the heterodimer. Approximately 71% of the ejaculated spermatozoa were immunolabeled after 30 minutes of incubation. In the controls, immunolabeling decreased from 71% at 0.5 hours to 49% by 10 hours (Figure 3, $P < .05$). By contrast, a 30-minute exposure to PSP-I/PSP-II resulted in an increase in the proportion of immunopositive spermatozoa compared to controls, with almost 100% of the cells immunolabeled ($P < .05$). This effect lasted during the whole incubation, maintaining around 82% of immunolabeled spermatozoa after 10 hours of preincubation with the heterodimer (Figure 3).

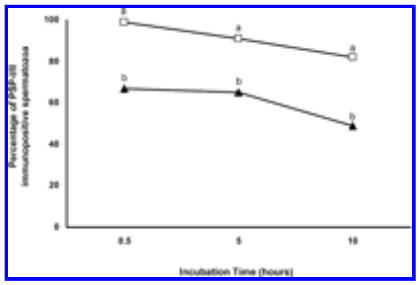

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Figure 3. Effect of the PSP-I/PSP-II heterodimer (□) (1.5 mg/mL in phosphate-buffered saline) on the percentage of immunolabeled ejaculated boar spermatozoa extended to a concentration of 1 x 10^6 spermatozoa/mL after 0.5, 5, and 10 hours of incubation at 38°C evaluated by light microscope. Controls (▲) were incubated in the absence of PSP-I/PSP-II heterodimer. Different letters indicate significantly different values ($P < .05$).

The SEM evaluation was performed to confirm the immunolabeling and its temporal localization on the plasma membrane head domains. The SEM disclosed several binding patterns which are presented in Figure 4: pattern a: PSP-I/PSP-II heterodimer overlying the entire acrosome domain (Figure 4a); pattern b: PSP-I/PSP-II heterodimer covering the acrosome domain with additional labeling on the postacrosomal area and a negative equatorial segment (Figure 4b); pattern c: slight immunolabeling of the postacrosomal area (Figure 4c); and pattern d: no immunolabeling of spermatozoa (Figure 4d). As expected, negative controls were totally immunonegative (Figure 4e), while positive controls had silver aggregates over the whole spermatozoon (Figure 4f). The PSP-I/PSP-II heterodimer was mainly located in the acrosomal area (>90%) of highly extended spermatozoa incubated either with or without heterodimer at 0.5 hours. During the incubation, there was a temporal redistribution of the pattern

of immunolabeling over time, from pattern a to pattern d. While most of the spermatozoa incubated without PSP-I/PSP-II (66%) showed pattern b after 5 hours, an increase in patterns c and d (26 and 30%, respectively) was observed after 10 hours of incubation. However, most spermatozoa incubated with PSP-I/PSP-II displayed pattern a throughout the 10-hour incubation period (Table).

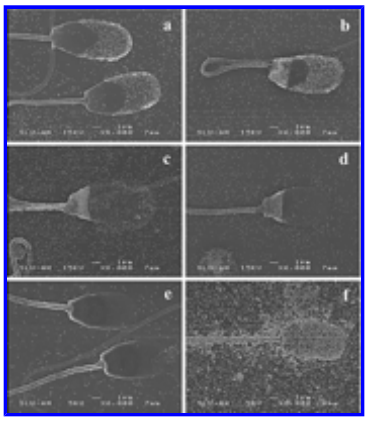

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Figure 4. Scanning electron micrographs of silver-enhanced immunogold-labeled highly extended boar spermatozoa incubated with a rabbit monospecific polyclonal antibody against PSP-II. The sperm population displayed 4 distinctive staining patterns. Pattern (**a**) PSP-I/PSP-II heterodimer labeling overlying the acrosomal sperm head domain. Pattern (**b**) PSP-I/II heterodimer labeling covering the acrosomal domain with additional labeling on the postacrosomal area. Pattern (**c**) immunonegative acrosomal domain and a slight staining of the postacrosomal area. Pattern (**d**) immunonegative sperm head. Negative and positive controls are shown in (**e**) and (**f**), respectively.

Discussion

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High dilution rates remain an inevitable consequence of some new biotechnologies, such as high-speed flow sorting of spermatozoa for chromosomal sex. Such high extension rates have been repeatedly documented as detrimental for spermatozoa, inducing changes in viability and motility patterns, all of which are primarily associated with the removal of adsorbed seminal plasma components (Maxwell and Johnson, 1999; Centurión et al, 2003; Caballero et al, 2004a). To avoid this undesirable effect, attempts to maintain the functionality of highly extended spermatozoa have included the addition of 10% (v/v) of SP or SP components of low molecular weight to the sperm suspensions (Ashworth et al, 1994; Maxwell et al, 1997).

Recently, we have identified the non-heparin-binding spermadhesin PSP-I/PSP-II heterodimer as one of the SP components responsible for such beneficial effect on boar spermatozoa (Centurión et al, 2003; Caballero et al, 2004b). However, to the best of our knowledge, whether this effect relates to the association of the heterodimer with the sperm plasma membrane has not been studied.

To evaluate the binding pattern of the PSP-I/PSP-II heterodimer on the spermatozoa, a rabbit monospecific polyclonal antibody against PSP-II was produced. In boar seminal plasma, spermadhesin PSP-II has been found exclusively associated into a heterodimeric complex with PSP-I, whereas PSP-I, which exists in excess over PSP-II, is present also as a noncomplexed molecular species (Calvete et al, 1995). Thus, the anti-PSP-II antiserum represents a specific immunochemical to detect the PSP-I/PSP-II complex. The immunocytochemistry clearly revealed that PSP-I/PSP-II heterodimer is located mainly on the acrosomal region of the head sperm and that the immunolabeling was being lost over time. Addition of exogenous PSP-I/PSP-II heterodimer caused, as expected, almost 100% of the spermatozoa to become immunolabeled. Contrary to a previous report suggesting that the PSP-I/PSP-II

complex lacked sperm-binding activity (Calvete et al, 1995), the present results show that a high subpopulation of highly extended boar spermatozoa (71%) did not entirely lose their adsorbed PSP-I/PSP-II coating in samples of highly extended ejaculated spermatozoa where no extra PSP-I/PSP-II was added (eg, controls). Similar results have previously been reported in boar spermatozoa in relation to the spermadhesins AQNs and AWN, which coat the sperm membrane after ejaculation in almost 100% of the spermatozoa but were progressively lost during incubation under capacitation conditions (Dostálová et al, 1994). In our experiment, the proportion of immunolabeled control spermatozoa dropped below 50% after 10 hours of incubation. However, the proportion of spermatozoa incubated in the presence of PSP-I/PSP-II heterodimer remained in about 80% positively labeled with the immunostain ($P < .05$). This indicates that 1) the heterodimer binds to the sperm membrane head domains before or at ejaculation and 2) the binding is intense enough to last for several hours in a large proportion of spermatozoa, even following an extreme dilution of the sperm suspension. However, the binding is probably not strong enough to remain unless an additional source of the heterodimer is present. Lastly, the highly extended, long-incubated spermatozoa are able to bind additional heterodimer and maintain functionality. Regarding functionality, while the percentage of membrane intact spermatozoa as well as the percentage of spermatozoa presenting appropriate mitochondrial activity were maintained during the incubation process, the percentage of progressively motile spermatozoa was always lower than that of membrane intact spermatozoa (and also below the proportions of immunolabeled spermatozoa). Differences among motility and mitochondrial activity have been reported earlier and probably reflect differences in sperm activity or the ability of the measuring methods (Windsor, 1997; Centurión et al, 2003). It has been recently hypothesized that mitochondria provide the sperm head and the midpiece with ATP (by oxidative phosphorylation) in order to maintain different gradient processes over the plasma membrane that are important for sperm survival. However, the ATP needed for sperm motility seem to be produced by glycolysis (anaerobic ATP production) by enzymes located in the fibrous sheath of the tail (Silva and Gadella, 2006). Although interesting as a hypothesis, there is a need for experimentally based proof for these assumptions.

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Redistribution of the different patterns of immunolabeling of PSP-I/PSP-II heterodimer on the surface of highly extended spermatozoa (1 x 10^6 spermatozoa/mL) after incubation either with or without PSP-I/PSP-II (exposed and nonexposed, respectively) during 0.5, 5, and 10 hours at 38°C

Although a higher percentage of motile cells was seen in the exogenous PSP-I/PSP-II group than in controls, the addition of PSP-I/PSP-II during incubation decreased their ALH when compared with control group. These results do not suggest that spermatozoa in PBS were hyperactivated, but indicate that the widths of the sperm heads' movement were significantly higher than those of spermatozoa in PSP-I/PSP-II. Consequently, the sperm motility should be more preserved in spermatozoa incubated in presence of PSP-I/PSP-II as the percentage of motile cells showed.

The percentages of acrosome-reacted and immunonegative spermatozoa were similar during the first stages of incubation. These results might indicate that the PSP-I/PSP-II heterodimer is linked to the acrosome while it remains intact. Nevertheless, it should be noted that when spermatozoa were incubated for 10 hours, a higher number of immunonegative spermatozoa than of acrosome-reacted spermatozoa was found. Moreover, in some of the spermatozoa, the PSP-I/PSP-II was partially (pattern b) or totally (patterns c and d) removed from the acrosome-intact sperm head membrane. These findings suggest that the removal of the heterodimer from the sperm acrosome domain occurs earlier

than the disruption of the acrosome and could, therefore, be related to remodeling of the sperm surface, as it occurs during the destabilization of the plasma membrane (Tulsiani et al, 1997). This hypothesis is in agreement with the fact that PSP-I/PSP-II seems to be removed from the sperm surface when spermatozoa are subjected to in vitro capacitation treatments (Caballero et al, 2004b). These data would suggest that, at least in vivo, PSP-I/PSP-II is released from the spermatozoa before reaching the ovulated oocytes and may not have the chance to play a role in gamete interaction (as do other SP proteins, such as the AWN [Rodríguez-Martínez et al, 1998]). However, the in vivo role of the heterodimer is yet to be determined.

The SEM study showed that PSP-I/PSP-II is a surface-adsorbed protein located principally on the acrosomal sperm head domain of ejaculated boar spermatozoa (pattern a). The PSP-I/PSP-II seems to migrate to the postacrosomal domain during the incubation process (patterns b and c), being finally lost from the sperm surface (pattern d). This redistribution is consistent with previous results in several species where it has been related to the processes of sperm capacitation and acrosome reaction (Kamaruddin et al, 2004; Barrios et al, 2005). However, before establishing a causal relationship between surface localization and temporal distribution to sperm capacitation, more studies are required. In any case, the electron microscopy showed an overwhelming population of spermatozoa (97%) with pattern a after 0.5 hours of incubation, which corresponded to samples showing the best sperm viability, motility and mitochondrial activity. Likewise, there was a clear trend toward redistribution to pattern d after 10 hours of incubation in a spermatozoa population (30%) whose viability, mitochondrial activity, and particularly motility were lowest.

Related to the protective effect of the heterodimer on boar spermatozoa, it could be argued that the PSP-I/PSP-II heterodimer would be responsible for the stabilization of the sperm membrane, thus counteracting destabilizing phenomena that lead to membrane deterioration, opening of the acrosome, and eventually cell death. Obviously, identifying such an additive for sperm suspensions resulting from flow cytometry should help to improve the efficiency of new biotechnological processes. Moreover, there is no negative influence of the heterodimer on the capability of fresh-extended boar spermatozoa to penetrate in vitro the homologous oocytes pre-exposed to low doses of PSP-I/PSP-II (Caballero et al, 2004b).

In conclusion, the protective effect of the PSP-I/PSP-II heterodimer on highly extended spermatozoa seems to last for at least 10 hours and could be related to the adhesion of the heterodimer to the acrosome domain of the sperm head plasma membrane. Although these results may indicate a stabilizing effect of the heterodimer on the fluidity of the sperm membrane and perhaps a decapacitating postejaculation role, further research is needed in order to clarify the causal link between the binding patterns of the PSP-I/PSP-II and the capacitation status of the spermatozoa.

Footnotes

Financial support was received from Ministerio de Educación y Ciencia (AGL2001-0471 and BFU2004-01432/BMC), Madrid, Spain, and SLF and Formas, Stockholm, Sweden.

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