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Boar seminal plasma exosomes: Effect on sperm function and protein identification by sequencing

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ABSTRACT

Mammalian seminal plasma contains membranous vesicles (exosomes), with a high content of cholesterol and sphingomyelin and a complex protein composition. Their physiological role is uncertain because sperm stabilization and activation effects have been reported. To analyze a putative modulatory role for semen exosomes on sperm activity in the boar, the effects of these vesicles on several sperm functional parameters were examined. Additionally, boar exosome proteins were sequenced and their incorporation into sperm was explored. Boar sperm were incubated under conditions that induce capacitation, manifested as increased tyrosine phosphorylation, cholesterol loss and greater fluidity in apical membranes, and the ability to undergo the lysophosphatidylcholine-induced acrosome reaction. After establishing this cluster of capacitation-dependent functional parameters, the effect produced by exosomes when present during or after sperm capacitation was analyzed. Exosomes inhibited the capacitation-dependent cholesterol efflux and fluidity increase in apical membranes, and the disappearance of a 14-kD phosphorylated polypeptide. In contrast, the acrosome reaction (spontaneous and lysophosphatidylcholine-induced) was not affected, and sperm binding to the oocyte zona pellucida was reduced only when vesicles were present during gamete coincubation. Liposomes with a lipid composition similar to that present in exosomes mimicked these effects, except the one on zona pellucida binding. Interaction between exosomes and sperm was confirmed by transfer of aminopeptidase activity. In addition, the major exosome protein, identified as actin, appeared to associate with sperm after coincubation. Exosome composition had a predominance for structural proteins (actin, plastin, ezrin, and condensin), enzymes, and several porcine seminal plasma-specific polypeptides (e.g., spermadhesins). Transfer of proteins from exosome to sperm and their ability to block cholesterol efflux supports a direct interaction between these vesicles and sperm, whereas inhibition of some capacitation-dependent features suggests a stabilizing function for exosomes in boar semen.

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1. Introduction

Mammalian sperm leaving the testis are morphologically differentiated, but immotile and unable to fertilize the oocyte. They must undergo several morphological and functional changes to become fully fertile. The first stage,

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known as maturation, takes place during sperm transit through the epididymis, where they experience an extensive plasma membrane remodeling that involves acquisition and redistribution and release of various components, including lipids and proteins. As a consequence of this process, sperm acquire progressive motility and a potential ability to recognize and fertilize an oocyte [1]. However, additional functional maturation steps must be completed for sperm to be able to fully express these capabilities. Sperm are stored in the terminal portion of the epididymis waiting for the appropriate signal that will cause their release at ejaculation. At that time, cells come into contact with accessory sex gland secretions and are deposited in the female reproductive tract, where they undergo several structural and functional changes that render them ready to find, recognize, penetrate, and fertilize an oocyte. This complex process is known as sperm capacitation and can be reproduced *in vitro* by sperm incubation under adequate conditions [1–3]. Capacitation is a complex cascade of molecular events that includes cholesterol efflux with the consequent modification of sperm membrane composition and fluidity [4,5], phospholipid scrambling [6], changes in intracellular ion concentrations [7], and increased tyrosine phosphorylation in several proteins [8]. The functional consequences of all these processes are reflected in the ability of sperm to undergo the acrosome reaction (AR), and acquisition of a distinctive pattern of motility known as hyperactivation [1].

Ejaculation and capacitation are intimately related, not only chronologically, but also functionally. It is assumed that accessory sex gland secretions stabilize sperm for their transit along the female tract. The ability of seminal plasma to prevent and revert capacitation was reported together with the description of this event [9]. This effect was later connected to inhibition of the induced AR [10,11] and tyrosine phosphorylation of sperm proteins [12]. Cholesterol was indicated as the probable cause, because it could reproduce the effects of seminal plasma [10,13].

Mammalian seminal plasma contains membranous vesicles (exosomes) characterized by a high cholesterol and sphingomyelin content, and a complex protein composition [14–18]. These vesicles are produced by the epididymis and the prostate [19]. Prostasomes, the membrane vesicles secreted by the human prostate, have been more extensively studied [20]. In addition, similar vesicles have also been isolated from the seminal plasma of rat, rabbit, ram, bull, stallion, and boar [16,18,21–24]. Because prostasomes have immunosuppressive, antioxidant, and antibacterial properties, it has been suggested that they are involved in several biological processes which can indirectly influence sperm function [20]. Regarding a direct action, it is known that human prostasomes can interact with sperm; however, the purpose and relevance of this interaction is still controversial, because activating and stabilizing effects have been postulated. Vesicles isolated from rabbit seminal plasma inhibit fertility [22]. Conversely, prostasomes were reported to promote forward motility of human sperm [25,26]. With regard to the AR, several groups studied the effect of prostasomes with diverse results [24,27–30]. Recently, it was reported that prostasomes can affect the tyrosine phosphorylation of sperm proteins [29,31]. However, a wide

study on the possible role of exosomes on different aspects of sperm function is still lacking. Only a few studies on the effects of exosomes on sperm capacitation are available, but none have been conducted in the boar.

In the present study, the effect of exosomes isolated from boar seminal plasma on cholesterol efflux, membrane fluidity, protein tyrosine phosphorylation, AR, and binding to oocytes were analyzed to determine a possible modulatory role for these vesicles on sperm function. Additionally, boar exosome proteins were identified by sequencing, and their incorporation into sperm was explored.

2. Materials and methods

2.1. Chemicals

All reagents used were of high purity or analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA), Fisher Scientific (Loughborough, Leicester, UK), Merck (Darmstadt, Hesse, Germany), or J.T.Baker (Phillipsburg, NJ, USA).

2.2. Samples

Semen samples were obtained by the standard gloved-hand technique from five adult hybrid boars (cross of three pure breeds: Large White, Pietrain, and Hampshire) housed at an artificial insemination center in the School of Veterinary Sciences of the University of Buenos Aires. Handling of animals was in accordance with the principles expressed in the "Legislation for the protection of animals used for scientific purposes" (European Commission).

Pre- and post- sperm-rich fractions were discarded, and the sperm-rich fraction was used for analysis. The following parameters were measured to determine semen quality: ejaculate volume, sperm viability, motility, concentration, morphology, and response in the hyposmotic swelling test. Only samples which met the following quality requirements were used: volume greater than 50 mL, progressive motility greater than 70%, abnormal sperm less than 20%, and concentration of at least 3×10^8 sperm per mL. Ejaculates were processed individually.

2.3. Sperm incubation

The sperm-rich fraction was diluted (1.5×10^7 cells per mL) in Tyrode's medium (100 mM NaCl, 3.1 mM KCl, 0.4 mM MgSO$_4$, 0.3 mM NaH$_2$PO$_4$, 5 mM glucose, 20 mM HEPES, 1 mM sodium pyruvate, 21.7 mM sodium lactate, 15 mM NaHCO$_3$ and 2 mM CaCl$_2$, pH 7.4) supplemented with 3 mg/mL BSA. Sperm were then incubated at 39 °C in a 5% CO$_2$ humidified atmosphere for up to 3 hours. To evaluate the effect of exosomes on different sperm functions, two experimental approaches were tested: vesicles were added either at the beginning or during the last 30 minutes of incubation. In a parallel set of experiments, sperm were incubated in a similar manner with liposomes with a lipid composition similar to exosomes. Sperm motility was estimated at the end of the incubation using a phase-contrast light microscope (magnification $\times$ 400) with a thermal stage (37 °C).

2.4. Acrosome reaction

In order to induce the AR, sperm (1.5×10^7 sperm per mL) were incubated with lysophosphatidylcholine (LPC; final concentration 100 μg/mL) for 30 minutes at 39 °C [32]. Cells were fixed with 4% formaldehyde in PBS for 1 hour at 4 °C, washed three times with 0.1 M ammonium acetate pH 9, placed on slides, and air-dried. Acrosomal status was determined after Coomasie Blue staining [33]. Briefly, sperm were permeabilized for 5 minutes in methanol and immersed for 2 minutes in 0.22% Coomasie Blue G-250 in methanol:acetic acid:water 50:10:40. After washing for 10 seconds with distilled water, slides were mounted using 90% glycerol in PBS. The acrosome reaction was quantified using light microscopy counting of at least 200 sperm per treatment (magnification × 400). The presence of a blue acrosome with a strong apical signal indicated an intact sperm, and those with lack of staining in the anterior head were considered acrosome-reacted.

2.5. Exosome isolation

The sperm-rich fraction was subjected to sequential centrifugation (800 × g for 20 minutes at room temperature and 10,000 × g for 30 minutes at 4 °C) to obtain seminal plasma free of sperm and cell debris. For vesicle isolation, the final supernatant was ultracentrifuged at 100,000 × g for 1 hour at 4 °C. The pellet was washed twice with 30 mM TRIS, 130 mM NaCl, pH 7.6, and centrifuged at 100,000 × g for 1 hour at 4 °C. After resuspension in 1 to 2 mL of this buffer, vesicles were purified by gel filtration on a Sephadex G-200 column (210 × 20 mm) pre-equilibrated with the same buffer. The void volume, containing exosomes, was centrifuged at 100,000 × g for 1 hour at 4 °C and the pellet resuspended either in Tyrode's medium for incubation with sperm or in PBS for protein studies [17]. Protein content was quantified according to Bradford [34].

2.6. Liposome preparation

Lipid vesicles with a composition similar to seminal plasma vesicles (645 μM cholesterol, 138 μM sphingomyelin, 110 μM di-palmitoylphosphatidylethanolamine, 57 μM di-palmitoylphosphatidylcholine, and 28 μM di-palmitoylphosphatidylserine) were prepared [16]. Stock solutions of each lipid (5 times concentrated in chloroform:methanol 2:1) were mixed in equal parts. Solvent was evaporated under nitrogen with continuous rotation to obtain a fine lipid layer. Liposome suspension was obtained by the addition of Tyrode's medium and sonication (three pulses of 1 minute, separated by intervals of equal length) in a Bransonic 1200 sonicator (Branson Ultrasonics Corporation, Danbury, CT, USA).

2.7. Electrophoresis

Protein extracts from exosomes or sperm incubated under different conditions were analyzed by SDS-PAGE. Cells were washed twice with PBS and resuspended in nonreducing Laemmli buffer (0.05 M TRIS, 0.5% SDS, 5% glycerol, pH 6.8). After heating for 5 minutes at 100 °C, samples were centrifuged at 10,000 × g for 2 minutes and the supernatants removed and stored at −20 °C until use. Before running, samples were supplemented with 5% β-mercaptoethanol and boiled for 5 minutes. After centrifugation, proteins (corresponding to 5×10^6 sperm per lane) were separated on 12.5% SDS-polyacrylamide gels. Exosomes were diluted in Laemmli buffer and denatured by heating for 5 minutes at 100 °C before running. To prepare exosome samples for sequencing, Laemmli buffer was supplemented with 10 mM dithiothreitol before boiling. After cooling, sulfhydryl groups were blocked by incubation with 20 mM iodoacetamide for 20 minutes at room temperature. Samples were analyzed using 7% or 12.5% polyacrylamide gels (9 μg protein per lane) [35]. Molecular weight standards were from Bio-Rad (Precision Plus, Dual color; Hercules, CA, USA). After electrophoresis, proteins were revealed by silver staining [36] or Western blot analysis.

2.8. Western blot

Gels were electroblotted to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) for 2 hours at 50 V and 4 °C. For immunoblotting, nonspecific binding sites on the membranes were blocked with 10% gelatin for 1 hour at room temperature. All incubation and washing procedures were carried out with PBS supplemented with 0.1% Tween 20 (PBST). After blocking, membranes were incubated overnight at 4 °C with a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, New York, NY, USA; clone 4G10) diluted 1:10,000 in blocking solution. After washing three times for 5 minutes with PBST, peroxidase-conjugated secondary antibody (Jackson Laboratories, Sacramento, CA, USA) diluted 1:10,000 in PBST containing 1 mg/mL BSA was added and incubation was done for 1 hour at room temperature. Membranes were extensively washed and immune complexes detected by enhanced chemiluminescence using ECL Plus (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) and Kodak Biomax Light Films (Kodak, Rochester, NY, USA).

2.9. Protein sequencing

Polypeptides identified after exosome analysis by SDS-PAGE and silver staining were cut and treated for in-gel digestion. Briefly, bands were destained with acetonitrile and ammonium bicarbonate buffer, and trypsin (porcine, modified, sequence grade; Promega, Madison, WI, USA) was introduced to the dried gel pieces. After overnight tryptic digestion, peptides were bound to a C18 column and eluted with acetonitrile. Mass lists were generated by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using an Ultraflex I TOF/TOF from Bruker Daltonics (Bremen, Germany). Identity searches were performed by scanning the NCBInr sequence database with the tryptic peptides using the current version of the search engine ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). The spectrum was internally calibrated using autolytic tryptic peptides, and the error was set at ± 0.03 Da. One missed cleavage was allowed, and methionine could be oxidized. The significance of the identity was judged from the search engine's scoring system and other parameters from the similarity between empiric and calculated peptide masses.

2.10. Isolation of sperm apical membranes

Apical plasma membranes were obtained by nitrogen cavitation [37]. Sperm suspensions (4.5×10^8 cells) were centrifuged (10 minutes at $800 \times g$) and the pellet resuspended in 8 mL of 5 mM TRIS, 0.25 M sucrose, pH 7.4. Cells were then placed in a Parr Bomb (Parr Instrument Company, Moline, IL, USA), equilibrated at a nitrogen pressure of 650 lb/in^2 for 10 minutes, and slowly extruded (over a 60–90-second interval) into a mixture of 1 mM EDTA, 0.2 mM phenylmethanesulfonic fluoride, and 1 mM sodium vanadate (kept at 0 °C). After sequential centrifugation ($1000 \times g$ for 10 minutes and $6000 \times g$ for 20 minutes) at 4 °C to remove cells and cellular debris, membranes contained in the supernatant were recovered by ultracentrifugation at $100,000 \times g$ for 30 minutes at 4 °C. The final pellet was resuspended in 50 μL of PBS.

2.11. Membrane fluidity

Membrane fluidity was determined by electron spin resonance (ESR) using 5-doxylstearic acid as a spin probe. Briefly, the probe (1 mM) was added to the sperm apical membrane preparation to achieve a 1:50 spin probe:phospholipid molar ratio. After a 10-minute incubation at 20 °C, ESR spectra were recorded using an X-band ESR Spectrometer Bruker ECS 106 (Brucker Instruments, Berlin, Germany). The spectrometer settings were: 3485 G center field, 100 G sweep width, 10 mW microwave power, 50 kHz modulation frequency, 0.203 G modulation amplitude, 40.96 ms conversion time, 655.36 ms time constant, 2×10^4 gain, and 1024 points resolution. Membrane fluidity was estimated by the order parameter S, which was calculated using the hyperfine constant values measured from the ESR spectrum ($A_{//}$ and $A_{\perp}$). Calculations and the correction of the $A_{\perp}$ value were performed as described [16]. The S parameter provides a measure of the degree of structural order in the membrane: an S value of 1 represents a rapid spin-label motion restricted to one axis, and S = 0 indicates a fast isotropic motion, i.e., maximum freedom. Accordingly, a decrease in the S value reflects increased membrane fluidity [38].

2.12. Oocytes

Porcine ovaries were obtained from an abattoir and frozen until use. After thawing and puncturing to induce follicle rupture, oocytes were isolated by filtration through nylon meshes of decreasing pore size (200, 174, and 54 μm) using 10 mM sodium phosphate, 130 mM NaCl, 2 mM ethylene glycol-bis acid (2 -aminoetileter)-N, N, N', N'-tetraacetic acid, 11 mM sodium citrate, pH 7.0 [39]. Isolated oocytes were washed by pipetting through several drops of buffer and stored at 4 °C in a solution of high ionic strength (0.5 M [NH$_4$]$_2$SO$_4$, 0.75 M MgCl$_2$, 0.2 mM ZnCl$_2$, 0.1 mg/mL polyvinyl alcohol, pH 7.4) until use [40].

2.13. Binding assays

Oocytes (8–10 per droplet) were extensively washed by pipetting through five droplets of Tyrode's medium (1 hour total time), placed in fresh medium supplemented with 3 mg/mL BSA, and incubated for 30 minutes at 39 °C in a 5% CO$_2$ humidified atmosphere. Oocytes were inseminated with sperm previously incubated for 3 hours under capacitating conditions (3×10^5 sperm per droplet). Sperm incubated in the presence of exosomes or liposomes either throughout the entire capacitation time, or only during the last 30 minutes, were used to evaluate the effect of these treatments on sperm ability to bind to the zona pellucida (ZP). Additionally, exosomes or liposomes were added to the incubation drop to analyze the effect of their presence during the binding assay. Drops were covered with mineral oil and incubated for 30 minutes at 39 °C. After coincubation, oocyte–sperm complexes were washed three times with medium to remove sperm not firmly bound to the ZP. Oocyte–sperm complexes were fixed with 0.1% formaldehyde for 5 minutes, washed three times with medium, and transferred to a drop of 2.3% sodium citrate:ethanol 3:1 containing 0.75 mg/mL of polyvinyl alcohol. Finally, oocyte–sperm complexes were incubated in 30 μg/mL Hoechst 33342 for 8 minutes at room temperature, washed twice with citrate-ethanol solution, and mounted with glycerol:2.3% sodium citrate 9:1. The number of bound sperm per oocyte was determined by fluorescence microscopy using a Nikon Optiphot Microscope (Nikon Corporation, Tokyo, Japan) at magnification $\times$ 200 [41].

2.14. Analysis of protein transfer from exosomes to sperm

To analyze the possible aminopeptidase transfer from exosomes to sperm, aliquots of semen samples containing 6.5×10^7 sperm were centrifuged at $800 \times g$ for 5 minutes to eliminate seminal plasma. Sperm were suspended in 600 μL of either capacitation medium (Tyrode's supplemented with 3 mg/mL BSA, pH 7.4), or 320 mM sucrose, 20 mM 2-(N-morpholino) ethanesulfonic acid pH 5. Mixtures were incubated 45 minutes at 39 °C in the absence and presence of exosomes (final concentration 0.25 mg protein per mL). After incubation, cell suspensions were centrifuged for 5 minutes at $800 \times g$ and sperm pellets were washed twice with PBS. Aminopeptidase activity was quantified in the cell pellet and in the original exosome sample. Results were expressed as the percentage of transferred enzyme (assuming activity in the vesicles as 100%).

In addition, protein transfer from exosomes to sperm was studied by analyzing the protein profile of sperm incubated with exosomes during capacitation by SDS-PAGE and silver staining. Sperm pellets washed twice with PBS before extraction or vesicles incubated without cells, were used as control samples.

2.15. Aminopeptidase activity

Aminopeptidase activity was determined by release of p-nitroaniline from the synthetic peptide Suc(Ala)$_3$pNA [42]. Samples were resuspended in 1 mL of aminopeptidase substrate solution (Suc(Ala)$_3$pNA 1 mM in buffer 0.2 M TRIS-HCl pH 7.8), incubated for 30 minutes at room temperature, and the amount of p-nitroaniline released was quantified by measuring absorbance at 410 nm. For

cells, absorbance was measured in the supernatant obtained after centrifugation at $800 \times g$ for 5 minutes.

2.16. Cholesterol determination

Cholesterol content in sperm apical membranes was measured using a Colestat kit (Wiener Laboratory, Rosario, Santa Fe, Argentina) [43].

2.17. Statistical analyses

Results were expressed as the mean ± SEM of five to eight experiments. Statistical analysis was performed using one-way ANOVA and the Newman–Keuls multiple comparison posttest for cholesterol and membrane fluidity data, two-way ANOVA and Bonferroni posttest for AR analysis, one-sample Student t test for binding assays, and the paired t test for aminopeptidase transfer. All statistical procedures were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

3. Results

Before analyzing the effect of exosomes on boar sperm tyrosine phosphorylation, we evaluated the pattern of phosphorylation before and during incubation in capacitating conditions. Unlike most mammalian sperm, fresh (e.g., nonincubated) boar sperm have tyrosine phosphorylated proteins (Fig. 1). Western blot analyses showed two major phosphorylated bands of 45 and 49 kD, and some additional polypeptides of 37, 41, 58, and 65 kD with a significantly weaker signal. There was also a pair of bands in the low molecular weight area, the most intense corresponding to a polypeptide of 14 kD. To analyze changes associated with capacitation, sperm were incubated in capacitating conditions and aliquots were collected at various intervals. Sperm incubation was associated with: (1) an increase in phosphorylation of a group of high molecular weight proteins (82, 87, and 98 kD); (2) the appearance of two new (20 and 32 kD) phosphorylated bands; and (3) the disappearance of low molecular weight phosphorylated polypeptides (Fig. 1). Changes in tyrosine phosphorylation were progressive for up to 3 hours of incubation; therefore, this interval was chosen for the next experiments. Slight differences in this pattern were observed, especially in the intensity of the 20 kD band, even in samples from the same boar.

To determine whether exosomes affect the tyrosine phosphorylation changes associated with sperm capacitation, vesicles were added to the medium in three doses (corresponding to final cholesterol concentrations of 0.64, 6.45, and 64.5 μM). Using an alternative approach to assess whether vesicles could act as a decapacitating factor [22], the highest dose was added 30 minutes before the end of sperm incubation. Disappearance of the 14 kD band, which occurs during the incubation in capacitating conditions, was partially inhibited by exosomes in a dose-dependent manner (Fig. 2). This effect was observed when the vesicles were present throughout the entire incubation, or only during the last 30 minutes. On the contrary, exosomes did not seem to affect the capacitation-associated increase in phosphorylation of the other bands, even when these were present from the beginning or only at the end of incubation.
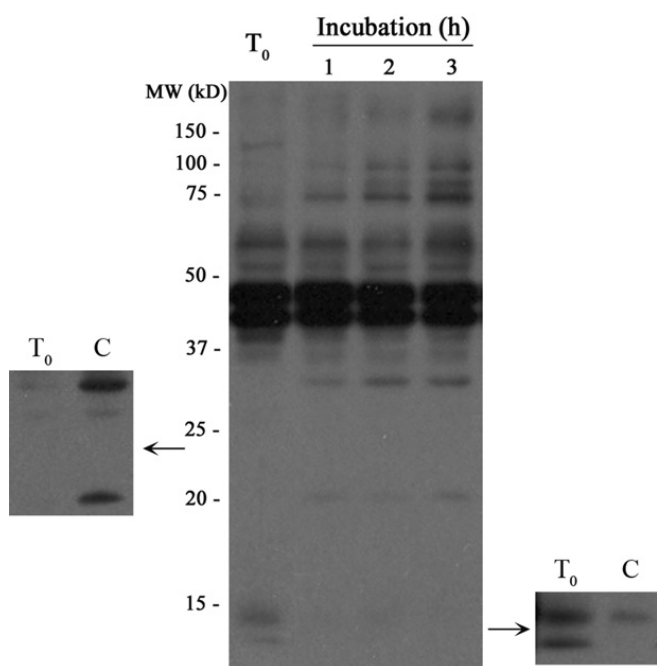

Fig. 1. Tyrosine phosphorylation of boar sperm proteins. Boar sperm were incubated in capacitating conditions for 3 hours. Sperm extracts were obtained before (T_0) or at various times (1, 2, and 3 hours) from start of incubation. Samples were analyzed using Western blot with an anti-phosphotyrosine antibody. Small panels at both sides correspond to longer exposure times for nonincubated (T_0) and 3-hour incubated sperm (C). MW, molecular weight.
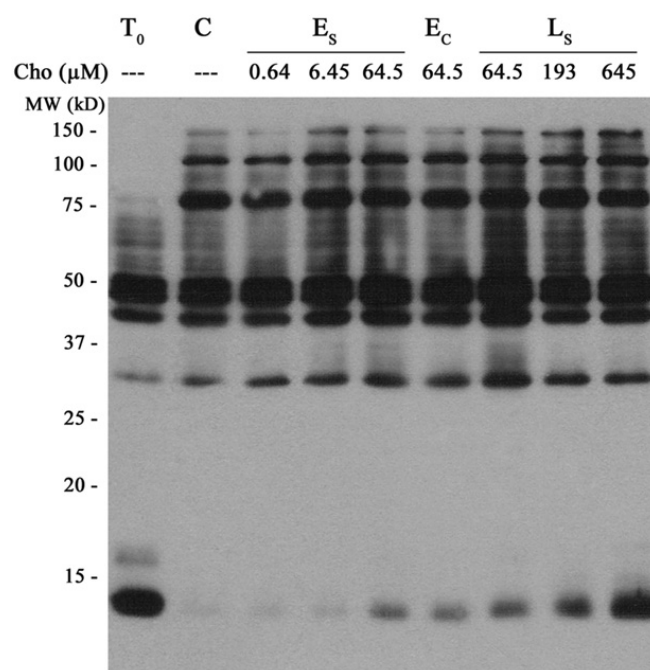

Fig. 2. Effect of exosomes and liposomes on tyrosine phosphorylation of boar sperm proteins. Sperm extracts were obtained before (T_0) and after 3-hour incubation in capacitating conditions in the absence (C) or presence of exosomes or liposomes from the beginning (E_S and L_S) or only during the last 30 minutes of capacitation (E_C). Cho, cholesterol; MW, molecular weight.

The increase in tyrosine phosphorylation that occurs during sperm capacitation has been associated with the loss of cholesterol [44]. To determine whether lipids contained in the exosomes could affect capacitation-associated changes in boar sperm phosphorylation, liposomes with a composition similar to that found in vesicles were used and similar results were obtained (Fig. 2). Because the inhibitory effect of cholesterol on tyrosine phosphorylation reported for sperm from other species was not observed, higher liposome concentrations were also tested (193.5 and 645 μM cholesterol). Although no change was detected in most phosphorylated proteins, there was a dose-dependent inhibitory effect on the signal of the 14 kD band (Fig. 2).

One of the functional consequences of capacitation is that sperm acquire the ability to respond to certain AR inducers. In the present study, LPC was used to induce the AR to differentiate the capacitating status of sperm subjected to the different treatments. Sperm incubated in capacitating medium for 3 hours had a significant increase in the rate of AR after treatment with LPC (28 ± 6% vs. 9 ± 2% for LPC vs. basal AR, P < 0.05; Fig. 3). In contrast, nonincubated sperm had similar levels of AR regardless of treatment (11 ± 1% and 5 ± 1% for LPC and basal AR respectively). The basal AR remained essentially at the same level for fresh and capacitated sperm (5 ± 1% vs. 9 ± 2%, respectively). Therefore, incubation conditions did not induce an increase in spontaneous AR, but allowed sperm to acquire the ability to respond to LPC.

In sperm incubated in the presence of vesicles (64.5 μM cholesterol; Fig. 3), the proportion of reacted sperm after treatment with LPC did not differ from control values (28 ± 6%), even when exosomes were present throughout capacitation (32 ± 4%) or during the last 30 minutes (25 ± 6%) (Fig. 3). Furthermore, vesicles did not affect the rate of spontaneous AR (10 ± 3% when included during the entire incubation and 8 ± 1% when present during the last 30 minutes, compared with 9 ± 2% in the absence of exosomes; Fig. 3). Therefore, exosomes did not affect spontaneous or LPC-induced AR *per se* and, moreover, did not interfere with acquisition of LPC sensitivity resulting from capacitation.

One of the most relevant events associated with sperm capacitation is the cholesterol efflux and the consequent increase in membrane fluidity that allows protein and lipid reorganization [4–6]. Because these changes occur mainly in the sperm head [37], apical membranes were obtained by cavitation and used to measure these two parameters. After incubation in capacitating conditions, there was a decrease in the cholesterol content (42 ± 4 vs. 27 ± 3 ng of cholesterol per 10^6 sperm for cells without and with incubation, respectively, P < 0.05; Fig. 4). This change in cholesterol content was reflected in a decrease in the order parameter S (0.680 ± 0.008 vs. 0.644 ± 0.005 for nonincubated and incubated sperm, respectively, P < 0.05; Fig. 4), indicating an increase in fluidity of sperm apical membranes.

The cholesterol content of sperm incubated in the presence of exosomes (43 ± 5 ng cholesterol per 10^6 sperm) was similar to that in noncapacitated cells (Fig. 4). In addition, the degree of membrane order (S = 0.665 ± 0.005) was also comparable with that in fresh sperm. Therefore, neither cholesterol loss nor an increase in membrane fluidity occurred in the presence of exosomes. When liposomes were included in the capacitation medium instead of exosomes, a similar result was obtained: neither cholesterol content (49 ± 6 ng per 10^6 sperm) nor membrane order and
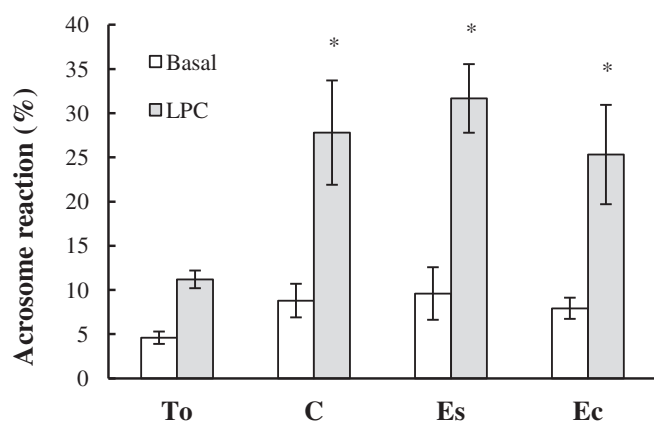
Fig. 3. Exosomes and acrosome reaction in boar sperm. Cells were analyzed before (To) and after 3-hour incubation in capacitating conditions in the absence (C) or presence of exosomes added from the start (Es) or after 2.5 hours of incubation (Ec). Acrosome reaction was quantified by Coomasie Blue staining in cells with (LPC) or without (Basal) stimulation with LPC. * P <0.05 versus basal. LPC, lysophosphatidylcholine.

Fig. 4. Cholesterol content and fluidity of boar sperm apical membranes. Nitrogen cavitation was used to obtain membranes from sperm before (To) or after 3-hour incubation in capacitating conditions in the absence (C) or presence of exosomes (Es) or lipids (Ls) (final cholesterol concentration = 64.5 μM). Membranes isolated after sequential centrifugation were used to measure cholesterol content (upper panel) and to determine the order parameter S (lower panel). * P <0.05.

fluidity ($S = 0.682 \pm 0.003$) differed from the value obtained for noncapacitated sperm (Fig. 4).

To study whether exosomes could be related to the ability of sperm to bind to the ZP, binding assays were carried out using sperm incubated in various conditions. The presence of vesicles during sperm capacitation did not modify ZP binding compared with control samples (Fig. 5). Results were similar when the vesicles were present during the entire sperm incubation ($108 \pm 9\%$ of control) or only during the last 30 minutes ($119 \pm 13\%$). In contrast, when exosomes were present not only during capacitation but also during the binding assay, the ability of sperm to bind to the ZP was impaired ($44 \pm 10\%$; $P < 0.05$). To determine whether this effect was related to the lipid nature of the vesicles, liposomes with an equivalent lipid composition were tested in the same conditions. Sperm–ZP binding was not modified when lipids were present throughout capacitation ($93 \pm 9\%$) or during the last 30 minutes ($93 \pm 19\%$). Unlike exosomes, the presence of lipids during the binding assay did not modify the ability of sperm to bind to the ZP ($82 \pm 17\%$).

To control for a possible effect of vesicles or liposomes on sperm motility, the rate of cells showing any motility pattern (total) and forward displacement (progressive motility) after a 3-hour incubation were quantified. There were no significant differences for any treatment (Table 1).

To detect an interaction between exosomes and sperm, the potential for transfer of vesicle-associated polypeptides to cells was evaluated. Vesicles isolated from seminal plasma have aminopeptidase activity, an enzyme that is absent in sperm, but can be acquired after incubation with vesicles [45]. The presence of aminopeptidase activity in boar exosomes and its absence in sperm were verified, and aminopeptidase was transferred from exosomes to sperm not only at pH 5.0 ($18 \pm 2\%$ of the activity added) but also at pH 7.4 ($5.5 \pm 0.5\%$).

Additionally, protein extracts from sperm incubated in capacitating conditions with or without exosomes were analyzed using SDS-PAGE followed by silver staining. In

Table 1
Total and progressive motility of incubated sperm.

Sperm capacitation conditions		Motility (%)	
Vesicles	Cholesterol (μM)	Total	Progressive
—	—	68 ± 3	51 ± 8
E_S	0.64	45 ± 10	40 ± 11
	6.45	45 ± 13	42 ± 10
	64.5	51 ± 5	41 ± 9
E_C	64.5	45 ± 11	44 ± 12
L_S	64.5	64 ± 10	62 ± 7

The ratio of motile sperm was evaluated after 3-hour incubation in capacitating conditions in the absence or presence of different amounts of exosomes included from the beginning (E_S) or only during the last 30 minutes (E_C). Liposomes with a lipid composition similar to exosomes included from the start of incubation were also tested (L_S).

addition to the original complex protein pattern of the sperm extract, a new band corresponding to the BSA contained in the capacitation medium was evident after sperm incubation (Fig. 6, asterisk). Extracts obtained from sperm incubated in the presence of exosomes had an additional band (42 kD; Fig. 6, arrowhead). Control treatments were carried out to exclude an experimental artifact and confirm the transfer of this polypeptide from vesicles to sperm. Sperm were washed twice with PBS before extraction or, in
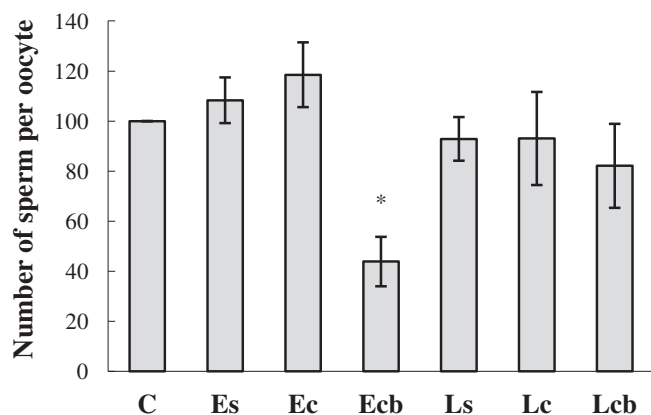
Fig. 5. Sperm–zona pellucida binding assays. Sperm were preincubated in capacitating conditions in the absence (C) or presence of exosomes (E) or liposomes (L) (64.5 μM final cholesterol concentration) and then coincubated with oocytes. Vesicles were added at the beginning (Es and Ls), after 2.5 hours of capacitation (Ec and Lc) or at the beginning and again in binding (Ecb and Lcb). The number of sperm bound per oocyte was quantified in each case and the results were normalized to the control (C) taken as 100%. ∗ P < 0.05.
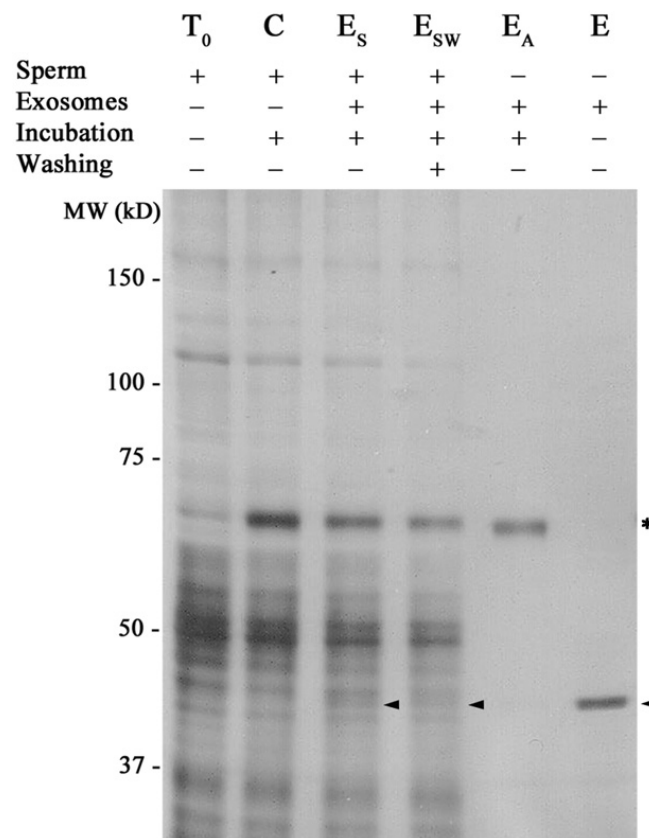
Fig. 6. Transfer of a polypeptide from exosomes to sperm. Extracts of sperm obtained before (T_0) and after incubation under capacitating conditions in the absence (C) or presence (E_S) of exosomes (64.5 μM cholesterol, 0.1 mg/mL protein) were analyzed by SDS-PAGE and silver staining. ∗ indicates BSA, and ◄ transferred protein. E, exosomes; E_A, exosomes incubated in the absence of sperm; E_{SW}, sperm incubated with exosomes and washed before extraction; MW, molecular weight.

an alternative approach, exosomes were incubated in capacitating conditions in the absence of sperm. Washed sperm still had the 42 kD additional band, whereas in the absence of sperm, only the band corresponding to BSA was visible (Fig. 6).

To analyze the protein composition of boar semen exosomes and identify the transferred polypeptide, extracts of these vesicles were separated by SDS-PAGE and proteins visualized using silver staining (Fig. 7). As expected, the protein pattern of exosomes was complex, with polypeptides between 10 and 150 kD, and a predominant band of 42 kD. Bands visualized after electrophoresis were excised for identification by MALDI-TOF mass spectrometry. There were structural proteins (actin, plastin, ezrin, condensin), enzymes (aminopeptidase, glyceraldehyde-3-phosphate dehydrogenase, aldehyde reductase, triosephosphate isomerase, hypoxanthine guanine phosphoribosyl transferase), intracellular chloride channels, and a number of pig seminal plasma proteins (spermadhesins: porcine seminal protein I, AQN and AWN) (Table 2). Predominant bands corresponded to cytoskeletal components, with the major 42-kD polypeptide identified as actin.

4. Discussion

The present study examined the putative modulatory effect of exosomes on boar sperm by investigating whether vesicles isolated from seminal plasma were able

to affect various sperm functional parameters related to capacitation.

One of the events associated with capacitation is the appearance and/or increase of tyrosine phosphorylation in sperm proteins. The identities of these proteins, and regulation of the phosphorylation process, differ among species. Noncapacitated boar sperm had a number of phosphorylated proteins (Fig. 1), similar to those previously reported [49–53]. Interestingly, a pair of bands in the low molecular weight area (approximately 14 kD) was observed. This low molecular weight region has not been analyzed to date, with the exception of a recent report of a 12-kD polypeptide [54].

Despite some minor variations among individual boars and samples, a common pattern of capacitation-dependent changes in the phosphorylation status of several polypeptides was observed: the appearance of two phosphorylated proteins of 20 and 32 kD, an increase in phosphorylation in a group of higher molecular weight bands (82, 87, and 98 kD), and the disappearance of phosphorylated lower molecular weight polypeptides (14 kD; Fig. 1). The signal reduction at 14 kD might be because of dephosphorylation or release of this protein from the sperm surface to the incubation media. Both possibilities are currently being explored. The increase in phosphorylation of the higher molecular weight proteins and the appearance of the 20- and 32-kD (p32) phosphorylated bands has already been described by most groups in experiments using boar sperm [49–53,55]. In addition, p32 has been identified as a tyrosine phosphorylated form of a proacrosin binding protein called sp32 [56]. The present study, however, detected the disappearance of a phosphorylated low molecular weight band during capacitation, which has not been previously reported.

The presence of exosomes during capacitation did not affect the increase in phosphorylation detected in several proteins at any of the concentrations studied. However, the capacitation-dependent disappearance of the 14-kD phosphorylated band was sensitive to the presence of vesicles (Fig. 2). Moreover, the degree of inhibition depended on the concentration of exosomes and was also observed when vesicles were present only during the last 30 minutes of sperm incubation. The putative modulation of sperm function by seminal plasma has been associated with proteins and lipids [10,57,58]. Consequently, the effect of liposomes with a lipid composition similar to exosomes on the tyrosine phosphorylation of boar sperm proteins was evaluated. Results were similar to those obtained with exosomes, namely an almost unaffected pattern of tyrosine phosphorylation, except for the concentration-dependent inhibition of the 14-kD band disappearance (Fig. 2). Therefore, the fate of the 14-kD protein, either its phosphorylation status or its presence on sperm, would be modulated by lipids.

The effects of seminal plasma, cholesterol, or vesicles on tyrosine phosphorylation have been previously studied in other species. In humans, whole seminal plasma blocked the capacitation-dependent tyrosine phosphorylation and also reverted the phosphorylation status of proteins in previously capacitated sperm [12]. The decapacitating ability of seminal plasma has usually been associated with cholesterol [10]. In the mouse, cholesterol sulfate inhibits the increase in
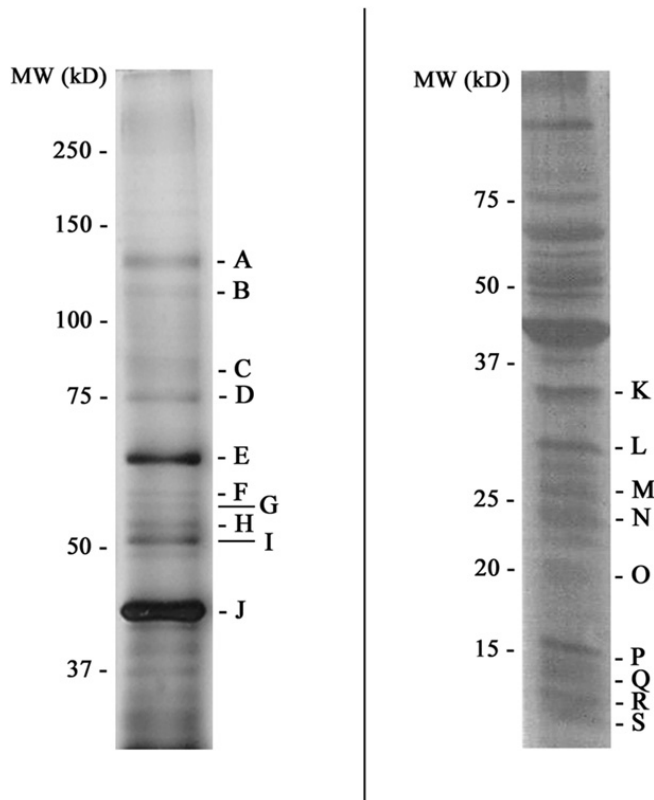

Fig. 7. Protein composition of exosomes analyzed by SDS-PAGE. Proteins were separated on 7% (left panel) and 12.5% (right panel) acrylamide gels followed by silver staining. Letters indicate bands selected for identification (Table 2). MW, molecular weight.

Table 2
Identification of boar exosome proteins.

Band	Protein identity	Accession number	Literature source
A	Filamin A	NP_056502	—
	Condensin	NP_055680	—
B, C	Myosin 1B (ß-actin)	NP_036355	[15,46]
D	Radixin/ezrin/villin and ß-actin	AAH47109	[14,15,46–48]
E	Plastin 3	P13797	[14,46,47]
F	Keratins	NP_001092053	[14]
G, H	Villin/ezrin/moesin/radixin	AAH68458	[15,46–48]
I	Plastin 3	AAH08588	[14,46,47]
J	ß-actin	AAA51578	[14,15,46–48]
	γ-actin	AAA56841	
K	Glyceraldehyde-3-phosphate dehydrogenase	DEPGG3	[14,15,46,47]
	Radixin (moesin B)	NP_001009576	[14,46,48]
	Aldehyde reductase	NP_99055	[15,47,48]
L	14-3-3 protein	NP_006752	[14,46,47]
	Chloride intracellular channel	XP_532079	[46]
M	Triosephosphateisomerase	AAB48543	[14,46,47]
	Hypoxanthine-guanine phosphoribosyltransferase	AAH04686	[14,46]
N	Ras-related v-ral simian leukemia viral oncogene homolog A	NP_112355	[15,46]
O	RAP1B Rs oncogene family	NP_056461	[14,15]
P	Cofilin	NP_0010044043	[15,46,47]
	ADP-ribosylation factor	NP_001649	[15,46]
Q	Peptidylprolylisomerase A	NP_999518	[46,47]
	PSP-I	NP_999002	—
	Spermadhesin AQN like protein	NP_998985	—
R	Calmodulin 1, 2, 3	NP_001734	[47]
	Spermadhesin AQN-3	AAB20129	—
S	Spermadhesin AQN-1	P26322	—
	Spermadhesin AWN-1	AAB21990	—

Gel slices containing bands isolated and detected after SDS-PAGE and silver staining were subjected to MALDI-TOF. Letters indicate the respective bands shown in Fig. 7.
Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PSP, porcine seminal protein.

tyrosine phosphorylation [44]. This same effect was reported for human sperm, although higher cholesterol sulfate concentrations were required [59]. Because approximately half of the seminal plasma cholesterol is associated with vesicles [27], these structures were proposed as the real effectors, and it was not until recently that this possibility was tested in the case of human prostasomes [29,31]. In the present study, unlike these earlier reports, the addition of vesicles or liposomes (even at a concentration equivalent to 645 μM cholesterol) had no effect on the capacitation-dependent increase in tyrosine phosphorylation of boar sperm proteins. The unique change observed in this study was the disappearance of the 14-kD polypeptide. Occasionally, a diminished pattern of tyrosine phosphorylation of sperm proteins was observed in the presence of exosomes. However, in all of these cases, there was a concomitant decrease in sperm motility, and those experiments were not considered (data not shown). Because major changes in sperm tyrosine phosphorylation are related to flagellar proteins, any change in the phosphorylation pattern should be accompanied by a parallel motility evaluation. This side-effect of exosomes on sperm motility might account for the different results between this study and others, which reported a vesicle-induced reduction in tyrosine phosphorylation, but failed to monitor motility or observe a detrimental effect on it [29,31].

Lipids play a decisive role in the structural and functional organization of the sperm plasma membrane. During capacitation, there is an entire reorganization of sperm membrane lipids in the apical area of the sperm head,

involving redistribution and cholesterol efflux [6]. Because fluidity of a membrane is highly dependent on its cholesterol content, these lipid changes increased membrane fluidity. Accordingly, cholesterol content and fluidity of apical membranes were analyzed as another parameter of sperm capacitation. Apical membranes were used to obtain better sensitivity, because cholesterol loss occurs mainly in this part of the sperm head, which represents a small proportion of the entire cell membrane. Incubation for 3 hours in capacitating medium reduced the cholesterol content of apical membranes by 35% (Fig. 4), a proportion similar to that reported for other species [60–62]. In agreement with this result, apical membranes from incubated sperm had greater fluidity than those isolated from nonincubated cells (Fig. 4). When sperm were incubated in the presence of either exosomes or liposomes, neither a decrease in cholesterol content nor an increase in membrane fluidity were observed (Fig. 4). Therefore, exosomes and liposomes blocked capacitation-dependent cholesterol loss and increased membrane fluidity. Although this possibility has been consistently proposed, the present results represent the first experimental evidence supporting this hypothesis. Moreover, to the extent of our knowledge, although the physical restraint of some capacitation-associated changes to the sperm acrosomal cap has been analyzed microscopically, the results presented in this report are the first quantitative analysis using apical membranes.

Another event clearly associated with capacitation is the acquisition of sperm sensitivity to undergo the AR when exposed to certain stimuli [1]. In our experimental

conditions, sperm incubated for 3 hours in capacitating medium underwent the AR in the presence of LPC, in contrast to nonincubated and nonstimulated cells. Four parameters supported the assertion that incubated sperm were capacitated: changes in tyrosine phosphorylation, ability to undergo the LPC-induced AR, reduction in cholesterol content, and an increase in membrane fluidity.

When the effect of exosomes on the ability of sperm to undergo the AR was evaluated, neither LPC-induced nor spontaneous AR were affected by any of the assay conditions (Fig. 3). A similar result was obtained when liposomes were used in place of exosomes (data not shown). Several groups have studied the effect of vesicles on the AR in diverse species [27,28,30,63]. However, results were contradictory and, in some cases, experimental conditions were not adequate. Regarding a spontaneous AR, in one study in boars, there was a slight increase when sperm were incubated in capacitating conditions in the presence of membrane vesicles [30]. On the contrary, no effect was reported in human sperm [63]. Regardless, a spontaneous AR in these two species is limited to a low proportion of cells and would not be an indicator of sperm functionality or capacitation. In the case of a stimulated AR, seminal plasma, prostasomes, and cholesterol inhibited the one induced by progesterone in human sperm [10,27]. In contrast, another study reported the increase of the progesterone-induced AR by prostasomes [28]. However, in this case, sperm were incubated with prostasomes at pH 5.5, a condition which could itself affect acrosomal structure. Conversely, it was reported that neither spontaneous AR nor calcium ionophore-induced AR was affected by incubation of boar sperm with liposomes [64]. Although LPC is not a physiological inducer of the AR, it is sensitive to the physiological state of sperm, because it is able to promote exocytosis only in capacitated cells, suggesting that the changes that occur with capacitation are necessary for LPC to induce the AR. However, when cholesterol loss was blocked by vesicles, sperm remained sensitive to LPC. Therefore, modification of cholesterol and membrane fluidity would not be the unique determinant of LPC sensitivity in boar sperm [65].

Capacitation is assumed to be associated with the exposure of and/or assembly of sperm receptors for the ZP. Consequently, the ability to bind to the ZP was taken as another sperm functional parameter. The presence of exosomes or liposomes throughout sperm capacitation or only during the last 30 minutes did not affect the ability of sperm to bind to the ZP. However, if exosomes were also present during the sperm–oocyte coincubation, sperm binding to the ZP was partially inhibited (Fig. 5). Considering that this effect was not observed when liposomes were used in lieu of exosomes, the inhibition would be related to the protein component of the vesicles.

Sperm motility was evaluated as an internal control to exclude the possibility that an indirect effect on this parameter caused ZP-binding inhibition. None of the treatments used in this study significantly modified the proportion of motile cells (Table 1). Although subtle changes in motility parameters that were not evident under the objective evaluation performed in this study cannot be excluded, that possibility was unlikely. Sperm

treated with exosomes or liposomes sporadically seemed to have more active movement (data not shown), in agreement with improved motility reported for prostasome-treated human sperm [25,26].

The diverse effects reported for cholesterol in sperm from humans, mice, and boars could be related to a number of causes, e.g., use of sperm at a different maturational step, treatment with cholesterol sulfate rather than cholesterol, and differences in the lipid content in sperm and seminal plasma. Cholesterol content and the cholesterol: phospholipid ratio are noticeably different between human and pig sperm [66]. Interestingly, when comparing species, boar sperm has the highest level of constitutive phosphorylated proteins and the lowest increase with capacitation. Some authors have suggested that cholesterol efflux is not as decisive in the case of the boar, and that sperm function could be regulated by a mechanism other than cholesterol content or membrane fluidity [53,67].

The direct interaction between exosomes and sperm proposed years ago has been well demonstrated by several groups, reporting not only the transfer of lipids but, most importantly, proteins from the vesicles to these cells [4,19,45,68–70]. In the present study, the possible interaction between exosomes and boar sperm was studied using two experimental approaches. When boar sperm were incubated with exosomes, a fraction of the vesicle-associated aminopeptidase was incorporated into sperm. This transfer occurred not only at acidic pH (5.0) but also, and more importantly, in more physiological conditions, i.e., in capacitation medium (pH 7.4). Additionally, cells incubated with exosomes had a 42-kD band in their protein profile that was absent in sperm incubated alone (Fig. 6). This protein matched the electrophoretic mobility of the most abundant protein present in exosomes. Transfer of this protein to sperm was verified when this band remained after cells were exhaustively washed, but was absent when vesicles were incubated alone. In order to identify this protein, and the entire profile of boar exosomes, polypeptides were separated by SDS-PAGE and sequenced by MALDI-TOF. The major component of boar exosomes was the 42-kD polypeptide identified as actin. This transfer of actin from vesicles to sperm would be supported by recent results obtained in cattle [71]. Although fusion of sperm with vesicles was reported many years ago, the precise mechanism involved in this interaction is not well understood [72,73]. The present results suggest that the cytoskeleton could be involved in the interaction between vesicles and sperm.

When the entire protein profile of boar semen exosomes was analyzed, the highest protein weight was associated with structural components (Table 2). Whether this composition is a consequence of the vesicles' origin, i.e., remnants of their secretion, or related to their function, remains to be determined. In addition to cytoskeletal proteins, polypeptides previously reported to be present in human prostasomes and/or epididymosomes from humans, bulls, and rams [14,15,46–48] were also detected (Table 2). Interestingly, spermadhesins were identified among the proteins present in boar exosomes. These components could explain the inhibitory effect of exosomes in sperm–ZP binding assays (Fig. 5), because porcine seminal protein I

and/or porcine seminal protein II spermadhesins have the ability to affect boar sperm interaction with homologous oocytes [74].

5. Conclusions

Exosomes inhibited apical membrane cholesterol loss and fluidity increase, and the disappearance of the 14-kD phosphorylated signal characteristic of boar sperm capacitation. Vesicles also reduced the ability of sperm to bind to the ZP when present during the interaction. However, no significant effects of exosomes on sperm motility, AR, or increase in tyrosine phosphorylation were detected. Transfer of two proteins from exosomes to sperm and the inhibition of cholesterol efflux by exosomes, suggests that there was a direct interaction between these structures and sperm. Taken together, our results suggest a stabilizing function for exosomes in boar semen.

Acknowledgments

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