Boar sperm tyrosine phosphorylation patterns in the presence of oviductal epithelial cells: in vitro, ex vivo, and in vivo models

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Abstract

Spermatozoa transport through the oviduct is a controlled process that regulates sperm capacitation. A crucial event involved in capacitation is protein tyrosine phosphorylation (TP). This study was undertaken to determine whether similarities exist in protein TP distribution between spermatozoa bound or unbound to oviductal epithelial cells (OEC) in three different conditions: i) in vitro, spermatozoa coincubated with OEC cultures; ii) ex vivo, spermatozoa deposited in porcine oviductal explants from slaughtered animals; iii) in vivo, in which sows were inseminated and the oviduct was recovered. The localization of phosphotyrosine protein was determined using indirect immunofluorescence. The distribution of protein TP was significantly (P<0.05) different between bound and unbound cell populations in all experiments. In sows inseminated close to ovulation, spermatozoa were found mainly in the utero–tubal junction, where spermatozoa exhibited a higher proportion of flagellum phosphorylation. Spermatozoa not bound to OEC exhibited high levels of protein phosphorylation (phosphorylated equatorial subsegment and acrosome and/or phosphorylated flagellum) in the ex vivo and in vivo experiments (P<0.05). However, unbound spermatozoa coincubated with OEC in in vitro conditions tended to show intermediate levels of TP (equatorial subsegment with or without phosphorylated flagellum). In spermatozoa bound to OEC, protein TP was located in the equatorial subsegment or presented no phosphorylation (P<0.05). Although sperm capacitation conditions in vivo were not reproducible in vitro in our experimental conditions, sperm and OEC binding seemed to be a mechanism for selecting spermatozoa with a low level of TP in in vivo, ex vivo, and in vitro experiments.

Introduction

After mating, millions of sperm cells are deposited in the female reproductive tract, where only a lesser number of these bind to oviductal epithelial cells (OEC) in the sperm reservoir (SR; Hunter et al. 1987). This interaction is a mechanism that allows selection of the optimal sperm population and the maintenance of fertilization competence (Hunter & Rodriguez-Martinez 2004). Sperm-OEC adhesion is mediated by molecules exposed on the sperm rostral surface, and carbohydrates localized on the oviductal cell plasma membrane in a species-specific manner (Green et al. 2001). Close to ovulation time, bound spermatozoa begin a progressive and continuous release due to endocrine changes that produce modifications in the plasma membrane of epithelial cells, secretory activity, and oviductal fluid composition (Fazeli et al. 1999, Georgiou et al. 2007).

During transit through the female genital tract, spermatozoa undergo a remodeling process known as capacitation, which provides spermatozoa with the ability to fertilize an oocyte (Austin 1951, Chang 1951). Capacitation is characterized by complex biochemical and biophysical changes that produce structural and morphological modifications in the sperm (Gadella 2008). Different events accompany the capacitation of mammalian spermatozoa, such as changes in intracellular ion concentration, plasma membrane fluidity, intracellular free calcium concentration, and protein tyrosine phosphorylation (TP; de Lamirande & Gagnon 1993, Visconti et al. 1995, Matás et al. 2011). The mechanism by which capacitation is controlled is not completely known, but the main factors that mediate this process are sterol acceptors, bicarbonate, and calcium (Harrison et al. 1996). Together, these signaling molecules activate the protein kinase A (PKA) and protein tyrosine kinase (PTK) pathways controlling protein phosphorylation (Visconti et al. 1995).

In several species, sperm capacitation is associated with an increase in protein TP (Visconti et al. 1995, Tardif et al. 2001, Pommer et al. 2003, Grasa et al. 2006). The presence of tyrosine-phosphorylated proteins in the flagellum suggests changes in motility parameters,
especially in the midpiece, which has been correlated with the acquisition of hypermotility (Nassar et al. 1999, Petrunkina et al. 2003). In addition, during capacitation, there is a redistribution of tyrosine-phosphorylated proteins localized in the acrosomal region of boar sperm (Flesch et al. 2001). These proteins may be involved in zona pellucida recognition and oocyte penetration (Dubé et al. 2005). Inside the equatorial segment in the head of mammalian spermatozoa, an area enriched in tyrosine-phosphorylated proteins, called the equatorial subsegment, has been described. It has been suggested that this region could be an organizing center involved in gamete fusion (Jones et al. 2003).

The adhesion between sperm and OEC allows the maintenance of sperm viability, motility, and a fertile life span because the oviduct modulates and controls sperm capacitation until the time of ovulation (Suarez et al. 1991, Töpler-Petersen et al. 2002). The main criterion for selective binding to the oviduct to ensure a suitable number of potentially fertile spermatozoa available for fertilization appears to be the uncapping status of the sperm population (Lefebvre & Suarez 1996, Fazeli et al. 1999). Other features, such as intact acrosomes, superior morphology, normal chromatin structure, low internal free calcium content, and reduced membrane protein TP, are necessary for binding (Ellington et al. 1998, Fazeli et al. 1999, Petrunkina et al. 2001, 2003, Gualtieri & Talevi 2003).

In vitro studies have shown that interactions between cultured OEC and sperm suppress and modulate TP protein status (Petrunkina et al. 2001, 2003, Zumoffen et al. 2010). We hypothesized that the sperm TP pattern is modulated by incubation with OEC and during the transit through the different sections of the oviduct in in vitro, ex vivo, and in vivo conditions. Our aim was to evaluate the distribution of TP protein in spermatozoa bound and unbound to OEC in three different experimental conditions: i) in vitro, with spermatozoa coincubated with OEC cultures during 1 h; ii) ex vivo, with spermatozoa deposited in porcine oviductal explants from slaughtered animals and incubated for 1 h; and iii) in vivo, in which sows were inseminated and the oviduct was recovered by surgery after 24 h.

**Materials and methods**

**Media**

Unless indicated, all chemicals used in this study were purchased from Sigma–Aldrich Química SA. Beltsville thawing solution (BTS) was used as seminal extender (Pursel & Johnson 1975). The medium used to capacitate spermatozoa was Tyrode Albumin Lactate Pyruvate (TALP; Rath et al. 1999). The culture medium for OEC (199-OEC) consisted of TCM 199 supplemented with 13% FCS, 150 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL).

**Sperm collection and preparation**

The sperm-rich fraction was collected from mature fertile boars using the gloved-hand method (King & Macpherson 1973). The semen was extended 1:2 with isothermal BTS. Sperm concentration, motility, acrosome integrity, and normal morphology were microscopically evaluated by standard laboratory techniques (Gadea & Mata’s 2000), selecting only high-quality semen for the experiments. After dilution in extender, a pool of semen from four different boars was used for all the experiments to avoid the effect of individual boars on the results.

The spermatozoa were washed through a two-step gradient of 45 and 90% iso-osmotic Percoll saline (Pharmacia) and centrifuged (700 g, 30 min) (Matás et al. 2011). The pellet was washed in TALP medium by centrifugation (700 g, 10 min) previously pre-equilibrated overnight at 38.5 °C in 5% CO₂ in 95% humidified air and with a final pH of 7.4. The remaining sperm pellet was resuspended in TALP medium. The concentration was adjusted according to the experiment.

**Sperm incubation with oviductal cells**

In vitro model: OEC culture

**Oviduct collection.** Oviducts from commercial cycling sows (Landrace × Large White) were collected in a local abattoir and transported to the laboratory in saline solution (0.9% w/v NaCl) containing 100 μg/ml kanamycin at 37 °C within 2 h post-mortem. In the laboratory, the oviducts were rinsed once with 0.04% cetrimide (alkyltrimethylammonium bromide) solution and twice with PBS supplemented with 4 mg/ml polyvinyl alcohol. The oviducts were trimmed of surrounding connective tissue and ovaries on Petri dishes under sterile conditions.

**OEC culture.** OEC were cultured as described by Romar et al. (2001). The oviducts were closed at one end with a clip, filled with a Trypsin–EDTA solution for endothelial cell culture (500 BAEE units of porcine Trypsin and 180 μg EDTA), closed at the other end, immersed in a Petri dish containing fresh solution, and incubated at 38.5 °C for 45 min. The cells used for culture were collected in a Petri dish after a second flush with fresh Trypsin–EDTA solution. The cell groups were dissociated by a repeated pipetting and transferred to a sterile conical centrifuge tube containing 199-OEC medium. Cell suspensions were centrifuged at 700 g for 5 min, the supernatant was discarded, and the pellet was resuspended in fresh OEC culture medium at a final concentration of 1 × 10⁷ cells/ml. One hundred microliters of the cell suspension were seeded into Petri dishes previously equilibrated for 2 h at 38.5 °C under 5% CO₂ containing 1900 μl of 199-OEC medium.

**Ethics**

The study was performed following approval by the Veterinary Ethical Committee of University of Murcia. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.
The culture medium was replaced every 48 h, until confluence was reached (typically after 6–7 days).

Spermatozoa and OEC co-culture. Petri dishes with a monolayer of OEC were washed under sterile conditions in TALP medium and spermatozoa were added (final concentration $1 \times 10^5$ cells/ml). Both were coincubated for 1 h (5% CO$_2$, 38.5 °C, and 95% humidified air). After this period, the medium with the unbound spermatozoa was collected with a pipette. Petri dishes with sperm bound to OEC were fixed in 2% p-formaldehyde/PBS for 60 min at 4 °C. Both bound and unbound sperm were processed and analyzed by immunocytochemistry to determine TP.

‘Ex vivo model’: oviductal explants

Oviductal explants and sperm coincubation. Oviducts were collected and handled as in the in vitro model. The oviducts used showed ovaries close to ovulation (presence of hemorrhagic corpus and preovulatory follicles). Sperm samples (0.5 ml) were deposited in the final section of the uterine horn (close to the utero–tubal junction (UTJ)) (1 $\times 10^5$ cells/ml), followed by clamping of the ends of the tract. Oviducts were incubated for 1 h at 38.5 °C in a humidified atmosphere of 95% air and 5% CO$_2$. Then, the oviduct was cut into four parts: ampulla, ampullary–isthmic junction (AIJ), isthmus, and UTJ, and unbound sperm were removed by washing with warm BTS and analyzed by immunocytochemistry.

Tissue sections and histological protocol. After collection of unbound sperm, the different oviductal pieces were fixed in Bouin’s solution for 24 h. Fixed tissues were washed repeatedly in 75% v/v methanol for 48 h. Samples were dehydrated, embedded in a paraffin block, and later sectioned transversely to a thickness of 10 μm. Sections were mounted on poly-l-lysine coated slides and deparaffinized. The samples were then processed by immunocytochemistry.

‘In vivo model’: artificial insemination and oviduct collection

Animals. Crossbred multiparous sows (Landrace×Large White) with an average parity number of 3 were used in the experiment. The animals were kept in individual pens in a temperature and humidity-controlled environment. Estrus detection was performed twice daily by experienced workers allowing sows to contact nose-to-nose with a mature boar and applying back pressure. The occurrence of estrus was defined by the standing reflex in front of a teaser boar, reddening, and swelling of the vulva. The sows were cervically inseminated (Import-vet S.A., Barcelona, Spain) at 12 and 24 h after the onset of estrus with $3 \times 10^9$ spermatozoa per dose in 80 ml BTS.

Oviduct recovery. The sows were anesthetized and laparotomized ~24 h after insemination (Tummaruk et al. 2007). Preoperative anesthetic and analgesic induction of animals was carried out by a combination of ketamine (100 mg/ml) 10 mg/kg (Imalgene 1000, Merial Laboratorios S.A., Barcelona, Spain), medetomidine (1.0 mg/ml) 0.2 mg/kg (Domtor, Pfizer S.A., Madrid, Spain), midazolam (5 mg/5 ml) 0.2 mg/kg (Dormicum, Roche), and morphine hydrochloride (20 mg/ml) 0.2 mg/kg (Morphine Braun 2%, B. Braun Medical S.A., Barcelona, Spain) administered i.m. The anesthetic level was maintained using isoflurane (Isoflo, Laboratorios Dr Esteve S.A., Barcelona, Spain) vaporized in oxygen at 2–3% (García-Vázquez et al. 2010). Ovaries and oviducts from animals were exposed through a mid-ventral incision. Oviducts were dissected and collected in Petri dishes. The number of follicles and corpora hemorrhagica for each ovary were recorded. After surgery, animals were killed by i.v. administration of sodium thiopental (10 mg/kg) (Pentotal Lab. Abbott S.A., Madrid, Spain).

Sperm recovery from the oviduct. The method of sperm recovery was a modification of the method described by Kunavongkrit et al. (2003) and others (Hunter 1981, 1984, Mburu et al. 1996). The oviducts on each side were tied and cut into four anatomical parts: ampulla, AIJ, isthmus, and UTJ. Every section was intraluminally flushed with 1 ml BTS at 37 °C from the cranial to the caudal section with a pipette. The fluid recovered was processed by immunocytochemistry assay. After flushing, every piece of the oviducts was fixed in Bouin’s solution for 24 h for further histological processing as described earlier.

Immunolocation of TP protein in spermatozoa

The localization of TP protein in spermatozoa was analyzed by indirect immunofluorescence, with a method described by Tardif et al. (2001) and adapted by Matás et al. (2011). Sperm suspensions from different experimental groups were washed with PBS and centrifuged (10 min, 270 g). Spermatozoa were fixed in 2% p-formaldehyde/PBS for 60 min at 4 °C. Samples were washed once in PBS and blocked with 2% (w/v) BSA–PBS and incubated overnight at 4 °C in a wet chamber. The samples were centrifuged again, resuspended in PBS, and spread on microscope slides. Then, the spermatozoa were incubated for 1 h at 4 °C with monoclonal anti-phosphotyrosine antibodies (4G10, Millipore, Temecula, CA, USA) at 1:200 dilution in 0.1% BSA/PBS. The slides were rinsed with PBS and incubated for an additional 1 h at 4 °C with FITC goat anti-mouse antibodies (Bio-Rad Laboratories) at 1:300 dilution in 0.1% BSA/PBS. After rinsing with PBS, coverslips were mounted on the slides with fluorescence medium (Dako, Carpinteria, CA, USA). Petri dishes and slides with histological samples were processed following a similar protocol.

Sperm were observed with a Leica DMR microscope equipped with fluorescent optics (excitation 450–490 nm; L3 filter) and bright field. Images were obtained using a microscope digital camera system (Zeis AxioCam HRc) and saved and edited using Axiovision Release 4.8 Software.

Experimental design

Three different experiments were designed to study the level and distribution of tyrosine phosphorylated protein in spermatozoa in contact with OEC.
In vitro model: effect of in vitro cultured OEC on sperm TP protein location

Sperm were incubated in the presence of in vitro cultured OEC. The sperm used in this experiment were previously selected by Percoll gradient simulating the sperm subpopulation that reaches the oviduct under physiological conditions (spermatozoa with normal morphology and without seminal plasma). Sperm selected by Percoll gradient but not incubated with oviductal cells were used as control. A total of eight replicates were made and 4705 sperm cells were analyzed (bound = 2352, unbound = 2353).

Ex vivo model: effect of oviductal explants on sperm TP protein distribution

In this second study, spermatozoa were deposited in porcine oviductal explants from slaughtered animals to analyze the influence of the environment of the different oviductal regions. The sperm used for this experiment were similar to those used in in vitro model. Four oviducts were used in this experiment, analyzing a total of 3574 spermatozoa (bound = 1320, unbound = 2254). Sperm selected by Percoll gradient but not incubated with oviductal explants were used as a control group (n = 799).

In vivo model: sperm TP protein location in sperm collected from oviduct after artificial insemination

Sows around ovulation were inseminated. The average parity number of sows in this experiment was 4.0 ± 1.5. The number of ovulations per sow was 11.0 ± 3.2.

Oviducts were recovered and the effect of epithelial cells and oviductal fluid composition on spermatozoa was studied. Sperm before insemination were used as a control. Eight sows were used for the experiment and a total of 2166 sperm were analyzed (bound = 19, unbound = 2147).

In all the three experiments, bound and unbound sperm were collected, processed, analyzed, and classified according to their TP protein pattern. Phosphorylation of the sperm proteins was detected in the acrosomal region, in the equatorial subsegment (triangular in appearance), and/or in the flagellum (Jones et al. 2008). For a better analysis, the eight possible combinations of TP signal in the spermatozoa were grouped according to localization into three different categories (Fig. 1):

Pattern I ‘low capacitation level’: spermatozoa with no fluorescence signal in the equatorial subsegment, with or without a signal in the acrosome region or the flagellum. Pattern II ‘medium capacitation level’: spermatozoa with a signal in the equatorial subsegment, no signal in the acrosome area, and with or without the presence of signal in the flagellum. Pattern III ‘high capacitation level’: this includes spermatozoa with signal in the equatorial subsegment and acrosome area and with or without the presence of signal in the flagellum. Image A shows fluorescence capture and image B the corresponding bright field image (100×) of different patterns of protein TP on boar sperm detected by indirect immunofluorescence. The arrows indicate different TP distribution: pattern I (PI), pattern II (PII), and pattern III (PIII). Scale bars, 10 μm.

Statistical analysis

The data were first examined using the Shapiro–Wilk test to assess normality distribution. In view of the non-Gaussian distribution of most of the data gathered, a non-parametric test was used. Data were expressed as mean ± S.E.M. and compared by a non-parametric Kruskal–Wallis test and a Conover–Inman test for all pairwise comparisons was used to compare groups of samples. Differences were considered statistically significant at P < 0.05.

Results

In vitro model: effect of in vitro cultured OEC on sperm TP protein location

The protein TP pattern changes when the sperm are incubated with OEC, especially as regard patterns II and III. As Table 1 indicates, bound and unbound sperm after OEC incubation showed less pattern II phosphorylation than non-incubated sperm (control group), although an increase in pattern III was observed in unbound sperm and a decrease in the same phosphorylation pattern in bound sperm compared with the control group. The distribution of fluorescence patterns was significantly different (P < 0.05) between bound and unbound cell populations (Table 1). The most common pattern of TP in OEC-bound sperm was pattern II (77.4%). OEC binding was most prevalent in sperm with non-phosphorylated...
heads and tails (Fig. 2), while higher proportions of subsegment and head and/or flagellum phosphorylated cells were found in the unbound population (36.4%). The lowest proportion of pattern IV was observed in sperm bound to OEC (17.9%) compared with unbound sperm (81.8%) and sperm not incubated with OEC (84.6%).

**Ex vivo model: effect of oviductal explants on sperm TP protein distribution**

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**Spermatozoa bound to the OEC**

The bound sperm with TP pattern I were more abundant in the UTJ than in other oviductal section (P<0.05) (see Table 2). In contrast, the fewest cells showing pattern II were observed in the UTJ. The percentage of sperm showing pattern III was similar in the isthmus, AIJ, and ampulla, but no sperm with this pattern were found attached to the UTJ. Finally, as regards pattern IV, there were no significant differences in the percentage of sperm attached to the isthmus, AIJ, and ampulla (37.0, 39.7, and 35.0% respectively), however, in sperm bound to the UTJ this proportion was the lowest (22.5%) (P<0.05).

When the different bound sperm TP patterns (I, II and III) were compared in each oviductal section, the highest proportion corresponded to pattern II, while patterns I and III were similar. In general terms, the proportion of patterns I and III was very low (or even non-existent in the case of pattern III in the UTJ) except for pattern I in the UTJ (close to 30%).

**Unbound spermatozoa recovered from oviductal lumen by flushing**

The patterns of fluorescence in unbound sperm are summarized in Table 2. The tyrosine-phosphorylated protein pattern I was similar in each section (P<0.05) and showed a low percentage (range 4.8–8.4%). The highest percentage of pattern II was observed in the AIJ (82.6%), while similar percentages of pattern II were found in the UTJ and ampulla sections (64.6 and 60.9% respectively). Flushed sperm samples displayed the highest percentage of pattern III in the isthmus (40.5%) and the lowest level in the AIJ (10.0%); the UTJ and ampulla showed similar percentages (30.4 and 30.5% respectively). Almost all the unbound sperm (75.7–85.6%) showed pattern IV.

When the different unbound sperm TP patterns were compared in each oviductal section, flushed samples showed a high percentage of pattern II in the UTJ, AIJ, and ampulla, which was different from the percentages shown by patterns I and III. Only in the isthmus section were sperm with pattern II and III observed in the same proportion. In general terms, very few pattern I sperm were observed in the different oviductal sections.

**In vivo model: sperm TP protein location in sperm collected from oviduct after artificial insemination**

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**Spermatozoa bound to the OEC**

Nineteen spermatozoa were found bound to the epithelial cells in 16 oviducts from 8 sows. No statistical analysis was made for this group because of the low number of spermatozoa found. Most of them were localized in the AIJ (n=8, 42.1%) and the isthmus (n=6, 31.5%); only two (10.5%) and three (15.7%) spermatozoa were found in the ampulla and the UTJ respectively.

With respect to the TP pattern, 84.2% of the sperm did not show any fluorescence signal (Fig. 3) and only three of them (two in UTJ and one in isthmus) showed equatorial subsegment TP (pattern II). Head phosphorylation was not found in sperm bound to OEC, which agrees with the data from the in vitro and ex vivo experiments. In contrast to the ex vivo experiment (oviductal explants), no flagellum TP was observed.

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**Table 1** Immunolocation of protein tyrosine phosphorylation patterns in boar sperm bound and unbound to OEC cultured *in vitro*. Data shown mean±S.E.M.

<table>
<thead>
<tr>
<th>Sperm source (n)</th>
<th>Bound (n)</th>
<th>Pattern I (%)</th>
<th>Pattern II (%)</th>
<th>Pattern III (%)</th>
<th>Pattern IV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not incubated sperm (1001)</td>
<td>No (2352)</td>
<td>3.5±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.8±3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.6±3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84.6±3.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Incubated sperm OEC (4705)</td>
<td>No (2352)</td>
<td>2.8±1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.7±4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.4±4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.8±3.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Incubated sperm OEC (4705)</td>
<td>Yes (2353)</td>
<td>18.4±3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.4±4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.1±1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.9±3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>n</sup>, number of spermatozoa evaluated; Pattern I, non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa; Pattern II, equatorial segment or equatorial segment and flagellum phosphorylated; Pattern III, equatorial segment and head and/or flagellum phosphorylated; Pattern IV, flagellum phosphorylation independent of phosphorylation at other locations. <sup>a,b,c</sup>Different letters in the same column indicate significant differences (P<0.05).
Table 2 (A) Distribution of protein tyrosine phosphorylation in boar spermatozoa bound to oviductal epithelial explants from different oviductal segments. (B) Protein tyrosine phosphorylation distribution in boar spermatozoa unbound to oviductal epithelial explants from different oviductal segments. Data shown mean ± S.E.M.

<table>
<thead>
<tr>
<th>Oviductal segment</th>
<th>n</th>
<th>Pattern I (%)</th>
<th>Pattern II (%)</th>
<th>Pattern III (%)</th>
<th>Pattern IV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-incubated sperm</td>
<td>799</td>
<td>4.6±0.7</td>
<td>88.3±1.1</td>
<td>7.0±0.9</td>
<td>80.3±1.4</td>
</tr>
<tr>
<td>UTJ</td>
<td>120</td>
<td>27.5±4.0a</td>
<td>72.5±4.0a</td>
<td>–</td>
<td>22.5±3.8a</td>
</tr>
<tr>
<td>Isthmus</td>
<td>400</td>
<td>8.0±1.3b</td>
<td>86.7±1.7b</td>
<td>5.2±1.4</td>
<td>37.0±2.4b</td>
</tr>
<tr>
<td>AIJ</td>
<td>400</td>
<td>5.7±1.1b</td>
<td>83.7±1.8b</td>
<td>10.5±1.5</td>
<td>39.7±2.4b</td>
</tr>
<tr>
<td>Ampulla</td>
<td>400</td>
<td>10.5±1.5b</td>
<td>81.0±1.9b</td>
<td>8.5±1.1</td>
<td>35.0±2.3b</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-incubated sperm</td>
<td>799</td>
<td>4.6±0.7</td>
<td>88.3±1.1</td>
<td>7.0±0.9</td>
<td>80.3±1.4</td>
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<tr>
<td>UTJ</td>
<td>82</td>
<td>4.8±2.3</td>
<td>64.6±5.0a</td>
<td>30.4±5.1a</td>
<td>84.1±4.0ab</td>
</tr>
<tr>
<td>Isthmus</td>
<td>614</td>
<td>7.6±1.0</td>
<td>51.7±2.0b</td>
<td>40.5±1.9b</td>
<td>85.6±1.4b</td>
</tr>
<tr>
<td>AIJ</td>
<td>766</td>
<td>7.3±0.9</td>
<td>82.6±1.3c</td>
<td>10.0±1.0c</td>
<td>75.7±1.5b</td>
</tr>
<tr>
<td>Ampulla</td>
<td>792</td>
<td>8.4±0.9</td>
<td>60.9±1.7ab</td>
<td>30.5±1.6a</td>
<td>81.1±1.3ab</td>
</tr>
</tbody>
</table>

n, number of spermatozoa evaluated; Pattern I, non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa; Pattern II, equatorial segment or equatorial segment and flagellum phosphorylated; Pattern III, equatorial segment and head and/or flagellum phosphorylated; Pattern IV, flagellum phosphorylation independent of phosphorylation at other locations. a,b,c Different letters in the same column indicate significant differences (P<0.05).

Unbound spermatozoa recovered from the oviductal lumen by flushing

A total of 2147 spermatozoa were collected and evaluated from the different parts of the oviduct. The highest number of spermatozoa was recovered from the UTJ section (1742). The proportion of each TP protein pattern in flushed sperm samples is represented in Table 3. In the UTJ, only 5.8% of spermatozoa showed fluorescence pattern I, which was lower than those in the other sections and in the control group (spermatozoa evaluated before insemination) (P<0.05). In the case of pattern II, no significant differences were observed when the isthmus, AIJ, and ampulla sections were compared (46.0, 36.0, and 43.5% respectively), all being lower than those in the UTJ (84.6%). No differences were found in the proportion of pattern III in the isthmus, AIJ, and ampulla, but, as in the case of pattern I, a low proportion (9.5%) of the spermatozoa observed in the UTJ had phosphorylated equatorial segment and head and/or flagellum (pattern III). When the sperm signal was classified according to the presence or absence of tyrosine protein phosphorylation in the flagellum (pattern IV), a lower percentage of sperm flagellum signal was observed in the isthmus (67.1%) than in the ampulla (85.5%), while intermediate values were found for the UTJ (76.4%) and AIJ (74.5%).

When the different unbound sperm TP patterns were compared in each oviductal section, the UTJ presented a higher percentage of pattern II than of patterns I and III. The isthmus represents the next section during sperm transit toward the fertilization site. Here, sperm pattern II was less common than in the UTJ, while pattern III increased (although not significantly different from pattern II). When the sperm were in the fertilization site (ampulla) or close to it (AIJ), patterns I, II, and III were present in the same proportions.

Discussion

The essential steps of capacitation are modulated in the caudal isthmus of the oviduct, where sperm are selected and stored in close contact with the epithelium. A crucial event involved in capacitation and the acquisition of fertilizing potential is protein TP (Töpfer-Petersen 1999). In this study, we analyzed different distribution patterns of protein TP in boar spermatozoa and their relationship with the ability to bind to OEC under in vitro, ex vivo, and in vivo conditions.

The results showed significantly (P<0.05) different patterns of protein TP in the sperm bound to epithelial cells in all experiments (in vivo, ex vivo, and in vitro), indicating a selective function in the interaction. Sperm after ejaculation showed very-low protein phosphorylation levels (Table 3), which only increased when the samples had been washed through Percoll gradients and the seminal plasma had been eliminated (Table 2). Fresh semen retains most of the seminal plasma proteins that stabilize the cells and prevent capacitation (Töpfer-Petersen et al. 1998). The molecules present on the sperm surface that come from seminal plasma, for example the spermadhesins AQN-1, AQN-2, and AWN, create a protective layer around the plasma

Figure 3 Tyrosine phosphorylation distribution in boar spermatozoa bound to OEC in the isthmus 24 h after insemination. Image A shows fluorescence captures and image B shows the corresponding bright field (100×). Scale bars, 10 μm.
membrane, stabilizing it. The cysteine-rich secreted proteins such as CRISP1 appear to be related to maturation processes and inhibit premature sperm capacitation (Udby et al. 2005). Incubation with 10% seminal plasma appears to be sufficient to inhibit TP, acrosome reaction, and several additional key steps in capacitation (Vadnais & Althouse 2011).

Several authors have demonstrated that unphosphorylated spermatozoa show a preferential binding ability to OEC after a short incubation period (Lefebvre & Suarez 1996, Fazeli et al. 1999). Furthermore, Petrunkina et al. (2001, 2003) demonstrated that long-term coincubations with oviducal cells maintain sperm viability and motility and slow the process of sperm membrane destabilization, when spermatozoa are bound to OEC. Spermatozoa attached to epithelial cells showed constantly low levels of protein TP (mainly pattern I), while unbound spermatozoa showed a high degree of phosphorylation (Yeste et al. 2009). In our study, not only spermatozoa bound to OEC cultures exhibited a low level of TP but also spermatozoa attached to the oviducts of inseminated sows before ovulation and preovulatory porcine oviducal explants presented this pattern (P<0.05).

We found that different TP patterns were associated with different sperm functions (Urner & Sakkas 2003). In spermatozoa bound to OEC, the protein TP was located in the equatorial subsegment or did not show any fluorescence. Such labeling may represent the first stage of phosphorylation during capacitation (Fig. 2). In boar testicular sperm, TP protein is distributed through the entire equatorial segment. During epididymal maturation, dephosphorylation and/or redistribution of phosphorylation occurs and the cauda spermatozoa exhibit only an intense triangular labeling in the posterior part of the acrosome and the equatorial subsegment (Jones et al. 2008, Fàbrega et al. 2011). SPAC1 and HSPA1A proteins have been determined in this event (Spinaci et al. 2005, Jones et al. 2008). The equatorial subsegment might be an organizing center that initiates membrane fusion during the fertilization process.

Phosphorylation in the acrosome, equatorial subsegment, and flagellum is related to an advanced stage of capacitation (Petrunkina et al. 2003). The presence of tyrosine-phosphorylated proteins in the flagellum is a preferential pattern of sperm capacitation in several species (Leclerc et al. 1997, Pommer et al. 2003, Grasa et al. 2006, Jagan Mohanarao & Atreja 2011). This phosphorylation is associated with a fibrous sheath, suggesting that it may be involved in hyperactivation (Pommer et al. 2003, Kumaresan et al. 2012b). Our data showed that spermatozoa washed through Percoll gradients and spermatozoa unbound to oviductal cells had higher proportions of flagellum phosphorylation (pattern IV, P<0.05) in all the experiments. Flagellum TP may be related to the release of bound spermatozoa from the epithelium (Lefebvre & Suarez 1996). However, these findings contrast with others that point to the recovery of faint labeling in the tail in boar spermatozoa (Petrunkina et al. 2001, Fàbrega et al. 2011). Equatorial subsegment and/or flagellum phosphorylation (pattern II) corresponds to an intermediate capacitation level (Petrunkina et al. 2001).

Acrosome phosphorylation was an unusual pattern and was not found in sperm bound to OEC in any of the three experiments. Suppressed acrosome phosphorylation may be a requirement for binding to oviductal cells (Petrunkina et al. 2003) because it represents an advanced stage of capacitation. Sp32 is a (pro) acrosin binding protein localized on the acrosome surface. During incubation in capacitating conditions, sp32 is phosphorylated and transformed into p32, a tyrosine phosphorylated protein (Dubé et al. 2005). These changes may affect the sperm's ability to bind to OEC. Moreover, during the acrosome reaction, phosphorylation in the acrosomal region decreased due to the redistribution of phosphotyrosine residues (Tardif et al. 2001, Dubé et al. 2005).

In vivo, boar spermatozoa are deposited in the female genital tract, pass through the UTJ, and accumulate in the isthmus of the oviduct, where a functional SR is established (Hunter & Rodriguez-Martinez 2004). The sperm distribution results showed that spermatozoa in sows inseminated around ovulation are mainly found in the UTJ where they exhibit a high proportion of flagellum phosphorylation. In contrast, in oviducal explants, spermatozoa were found along the oviduct in the same proportion. The UTJ is a physical barrier that controls

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**Table 3** Proportion of tyrosine phosphorylation patterns in boar spermatozoa recovered from different parts of the oviduct of sows 24 h after insemination and before insemination. Data shown mean±s.e.m.

<table>
<thead>
<tr>
<th>Oviductal segment</th>
<th>n</th>
<th>Pattern I (%)</th>
<th>Pattern II (%)</th>
<th>Pattern III (%)</th>
<th>Pattern IV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm ejaculated</td>
<td>301</td>
<td>63.4±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.5±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.9±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>UTJ</td>
<td>1742</td>
<td>5.8±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84.6±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.4±1.0&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isthmus</td>
<td>152</td>
<td>21.7±3.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>46.0±4.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.2±4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.1±3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AIJ</td>
<td>122</td>
<td>26.2±3.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.0±4.3&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>37.7±4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.5±3.9&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ampulla</td>
<td>131</td>
<td>21.3±3.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.5±4.3&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>35.1±3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.5±3.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>n</sup>, number of spermatozoa evaluated; Pattern I, non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa; Pattern II, equatorial segment or equatorial segment and flagellum phosphorylated; Pattern III, equatorial segment and head and/or flagellum phosphorylated; Pattern IV, flagellum phosphorylation independent of phosphorylation at other locations. <sup>a,b,c</sup> Different letters in the same column indicate significant differences (P<0.05).
the quality and quantity of spermatozoa present in the oviduct. Only sperm cells with an intact acrosome, low internal free calcium content, reduced membrane protein phosphorylation, and normal chromatin structure are able to bind to OEC (Ellington et al. 1998, Fazeli et al. 1999, Petrunkina et al. 2001, Gualtieri & Talevi 2003). In our study, unbound sperm exhibited a high percentage of protein TP in the equatorial subsegment, acrosome, and flagellum (P < 0.05). Kumaresan et al. (2012a) speculated that an early increase in the population of spermatozoa with such a phosphorylation pattern would increase the chances of fertilization. The adhesion between sperm and OEC is mediated by molecules exposed on the sperm rostral surface and carbohydrates localized on the oviductal cell plasma membrane in a species-specific manner (Green et al. 2001). Capacitated sperm lose binding sites during capacitation, and our in vivo results showed that only sperm with unphosphorylated heads are able to bind to cells.

At the time of ovulation, changes in the oviductal environment occur, promoting the ascent of sperm cells toward the ampulla and the site of fertilization (Hunter 1981). Recent studies have shown that the arrival of spermatozoa initiates a mutual sperm–epithelial signaling dialog (Fazeli et al. 2004). In an in vivo experiment, a small number of spermatozoa were seen bound to OEC around the ovulation stage. Most spermatozoa were in the lumen of the oviduct with a high level of TP, especially when the sperm were close to the fertilization site. Spermatozoa modified protein synthesis and oviductal fluid composition remodeling the binding sites in oviductal cells due to the production of a gradient of calcium during periovulatory moments (Gadella & Harrison 2000). In sows, several de novo synthesized proteins have been identified, and oviduct-specific glycoprotein is related to increased fertilization rates and polyspermy control (Coy et al. 2008).

Porcine oviductal fluid in the follicular phase promotes sperm viability and acrosomal integrity, decreases plasma membrane fluidity, and increases zona binding and polyspermy during IVF (Coy et al. 2010). However, other authors observed no differences in sperm motility during incubation except at 2 h, when spermatozoa incubated with preovulatory fluid showed higher motility (Kumaresan et al. 2012b). In the same study, preovulatory oviductal fluid induced TP in a higher proportion of boar spermatozoa than post-ovulatory fluid (Kumaresan et al. 2012b). These contradictory results may be related to the boar in question, incubation time, or oviductal fluid treatment. Our results showed that unbound spermatozoa exhibited high levels of tyrosine protein phosphorylation (patterns II and III) in all the experiments.

The final objective of the capacitation process is fertilization. Spermatozoa incubated with OEC cultures showed higher penetration and monospermy rates when short time periods were used (Hunter 1981). Moreover, the fertilization and cleavage rates of spermatozoa unbound to oviductal cells culture were lower than those for spermatozoa attached to the OEC (Kon et al. 2009). These results indicate that the ability of spermatozoa to bind to oviductal cells is related to their fertilizing ability.

In conclusion, sperm capacitation conditions in vivo are not reproducible in in vitro models under our experimental conditions. However, sperm-OEC binding was seen to be a mechanism for selecting sperm populations with a low degree of TP, as demonstrated in the in vivo, ex vivo, and in vitro experiments. Further studies are required to obtain more information about the mechanism by which the oviduct modulates sperm capacitation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Fundación Séneca 08752/PI/08 and MICINN-FEDER (AGL 2009-12512-C02-01-02).

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Received 15 April 2013
First decision 23 May 2013
Revised manuscript received 3 July 2013
Accepted 15 July 2013