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Oviduct Binding and Elevated Environmental pH Induce Protein Tyrosine Phosphorylation in Stallion Spermatozoa[1]

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ABSTRACT

Sperm-oviduct binding is an essential step in the capacitation process preparing the sperm for fertilization in several mammalian species. In many species, capacitation can be induced in vitro by exposing spermatozoa to bicarbonate, Ca^{2+}, and albumin; however, these conditions are insufficient in the horse. We hypothesized that binding to the oviduct epithelium is an essential requirement for the induction of capacitation in stallion spermatozoa. Sperm-oviduct binding was established by co-incubating equine oviduct explants for 2 h with stallion spermatozoa (2×10^6 spermatozoa/ml), during which it transpired that the highest density (per mm^2) of oviduct-bound spermatozoa was achieved under noncapacitating conditions. In subsequent experiments, sperm-oviduct incubations were performed for 6 h under noncapacitating versus capacitating conditions. The oviduct-bound spermatozoa showed a time-dependent protein tyrosine phosphorylation response, which was not observed in unbound spermatozoa or spermatozoa incubated in oviduct explant conditioned medium. Both oviduct-bound and unbound sperm remained motile with intact plasma membrane and acrosome. Since protein tyrosine phosphorylation can be induced in equine spermatozoa by media with high pH, the intracellular pH (pH_i) of oviduct explant cells and bound spermatozoa was monitored fluorometrically after staining with BCECF-AM dye. The epithelial secretory cells contained large, alkaline vesicles. Moreover, oviduct-bound spermatozoa showed a gradual increase in pH_i, presumably due to an alkaline local microenvironment created by the secretory epithelial cells, given that unbound spermatozoa did not show pH_i changes. Thus, sperm-oviduct interaction appears to facilitate equine sperm capacitation by creating an alkaline local environment that triggers intracellular protein tyrosine phosphorylation in bound sperm.

donkeys, equids, equine, horses, oviduct, pH, protein tyrosine phosphorylation, sperm capacitation, zebras

INTRODUCTION

During natural mating, mammalian spermatozoa are deposited in the female reproductive tract and subsequently migrate to a sperm reservoir, which, in many species, is located in the isthmus of the oviduct [1–3], although there are exceptions, such as the dog [4, 5]. In the sperm reservoir, spermatozoa bind via the apical region of the head to oviduct epithelial cells (OECs) [6–8]. In cattle and pigs, only spermatozoa with intact acrosomal and plasma membranes are able to bind to the oviduct [9–11]. After a period of association with the oviduct epithelium, the bound spermatozoa undergo essential capacitation steps and are released from the reservoir. The released spermatozoa have thereby acquired a hyperactive motility pattern that helps them to move through the extracellular matrices of the cumulus complex and zona pellucida in order to reach and fertilize the mature oocyte [1–3]. Despite being first described in 1951 [12, 13], the capacitation process is still not fully understood, although it is known to involve a series of changes, including reorganization of the sperm plasma membrane that facilitates cholesterol loss. These membrane events are induced by an increase in intracellular HCO_3^- concentrations and activation of second messenger systems, including a soluble adenylyl cyclase (sAC) and a rise in intracellular Ca^{2+} [14–16]. The activation of sAC and concomitant production of cAMP also result in the activation of protein kinase (PK) A, which, in turn, phosphorylates tyrosine residues on sperm proteins [15, 17, 18]. The necessity for cAMP-dependent protein tyrosine phosphorylation, especially in the sperm tail, is, in various species, related to the acquisition of hyperactivated sperm motility, and is considered to be a marker for some essential elements of the capacitation process [19–24]. The rapid cAMP-driven membrane changes also enable depletion of cholesterol, which, in turn, allows aggregation of lipid ordered microdomains at the apical ridge area of the sperm head [25]. These microdomains contain functional zona pellucida-binding protein complexes [26] and the SNARE proteins required to form the trans-SNARE complexes required for the docking of the outer acrosomal membrane to the apical sperm plasma membrane [27, 28]. In short, the cAMP-driven membrane changes allow albumin-dependent removal of cholesterol, which is followed by a slower series of functional membrane changes required for the acquisition of fertilizing potential.

While in vitro capacitation and fertilization are considered to be routine procedures in many species, and despite promising results in equine intracellular sperm injection (ICSI) programs [29–31], there are still no reliably successful conventional in vitro fertilization (IVF) protocols for equids

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[32–34]. Most likely, this is because functional stallion sperm capacitation is not induced in vitro using routine capacitation media (i.e., media containing bovine serum albumin [BSA], bicarbonate, and Ca^{2+}) to promote sperm capacitation as described for various other mammalian species, including the mouse [19, 20], pig [14], and human [35]. In cattle, induction of capacitation is enhanced by the additional use of heparin and phosphodiesterase inhibitors [36, 37]. The disappointing protein tyrosine phosphorylation response after incubating stallion spermatozoa in simple in vitro capacitation media can be compensated by addition of membrane-soluble cAMP analogs in combination with phosphodiesterase inhibitors, which combine to increase PKA activity and induce protein tyrosine phosphorylation in the tail of approximately 50% of stallion spermatozoa [38]. Alternatively, reactive oxygen species will induce protein tyrosine phosphorylation in stallion spermatozoa [39]. Recently, an increase in the alkalinity of the capacitation medium (to approximate pH values of 8) has been reported to induce protein tyrosine phosphorylation [40, 41]. However, it is unlikely that such a high pH is the physiological trigger involved in sperm capacitation in vivo, and it remains unclear how protein tyrosine phosphorylation and related sperm hyperactive motility are elicited physiologically in stallion sperm. The current consensus is that the central event in sperm capacitation in vivo is binding of the sperm to the oviduct. After a period of sperm-oviduct binding, the spermatozoa are released from the epithelium, by which time they have acquired the competence for hyperactive motility [42]. Despite the likely involvement of oviduct interaction in sperm capacitation in situ, during IVF the treatments commonly used to trigger capacitation are limited to removal of seminal plasma and incubation in a bicarbonate-, Ca^{2+}-, and albumin-enriched medium; this condition fails to elicit reliable IVF using equine gametes. It is, therefore, tempting to speculate that equine sperm-oviduct interaction is an essential requirement for equine sperm capacitation. While in situ sperm oviduct interactions are difficult to monitor and interpret, OEC monolayers have been used as a model system to study sperm-oviduct binding. Bovine, equine, and porcine spermatozoa have all been shown to exhibit a capacitation-specific chlortetracycline (CTC) staining pattern when released from homologous OEC monolayers [9, 43, 44]. However, the utility of oviduct monolayers to induce a more physiological sperm capacitation has been questioned, because OECs rapidly dedifferentiate during culture [11], and it has, therefore, been suggested that an equine oviduct explant model may be more representative of the in vivo situation [45]. In cattle, a similar system has been shown to activate spermatozoa, which, when released, have acquired the competence to fertilize an oocyte [46]. It has been further suggested that spermatozoa are activated/capacitated during the late preovulatory period when the oviductal microenvironment changes in a way thought to be relevant for inducing sperm capacitation and the release of activated spermatozoa [1]. The aim of the current study was, therefore, to develop an equine oviduct explant system and determine its ability to trigger essential steps in the capacitation of stallion spermatozoa. We hypothesized that binding to the oviduct epithelium is an essential requirement for the induction of capacitation in stallion spermatozoa. To this end, we investigated whether stallion spermatozoa have affinity for mare oviduct epithelial explants and whether this results in intracellular pH (pH_i) changes, protein tyrosine phosphorylation, and subsequent release of spermatozoa with hyperactivated motility. These new insights may help to explain why conventional IVF still fails in the horse.

MATERIALS AND METHODS

Chemicals and Reagents

Propidium iodide (PI), SYBR14 (LIVE/DEAD Sperm Viability Kit), JC-1, Hoechst 33342, BCECF-AM, Alexa Fluor 488-conjugated goat anti-mouse antibody, and Texas red-conjugated goat anti-rabbit antibody were obtained from Molecular Probes (Ghent, Belgium). Monoclonal 4G10 Platinum anti-phosphotyrosine mouse antibodies were obtained from Millipore (Overijse, Belgium). Triton X-100, PNA-FITC, the rabbit anti-tubulin antibody, fatty acid-free bovine serum albumin (A9418; cell culture tested), and all other chemicals not otherwise listed were obtained from Sigma-Aldrich (Bornem, Belgium).

Animals

Oviducts were collected at a slaughterhouse soon after the slaughter of healthy Warmblood mares aged 5–22 yr and without any visible reproductive tract pathologies. Only oviducts from mares with a large follicle (>35-mm diameter) in combination with estrous edema in the uterine wall, indicating imminent ovulation, were used for this study.

Preparation of Oviduct Explants

Five oviducts per experiment were prepared as previously described by Nelis et al. [45]. Briefly, oviducts from preovulatory mares were dissected free of excess connective tissue, clamped at both ends, and transported on ice in sterile 0.9% saline containing 50 μg/ml gentamycin. On arrival at the laboratory, the oviducts were washed in PBS and the epithelial cells harvested by scraping the mucosa at the ampullary-isthmic junction of the longitudinally incised oviduct. The harvested cellular material was transferred to a tube containing Hepes-buffered Tyrode albumin lactate pyruvate medium and left to settle for 10 min, after which the cell pellet was resuspended in 3 ml of fresh Hepes-buffered Tyrode albumin lactate pyruvate washing medium. The process of sedimentation was repeated twice. Afterwards, the harvested cellular material was washed and cultured overnight in Dulbecco modified Eagle medium/nutrient mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) at 38.5°C in a humidified atmosphere of 5% CO_2 in air. The timespan from slaughter of mares to seeding of the cells was approximately 3–4 h.

Semen Collection and Preparation

Semen was collected using an artificial vagina (Colorado State University) from three adult stallions of proven good fertility. The raw ejaculate was filtered through gauze to remove the gel fraction and any debris before visual evaluation of sperm motility by light microscopy (200×) on a heated stage at 37.0°C; assuming good motility, the semen was immediately transported to the laboratory for further processing. Fresh semen (1 ml) with a concentration of 100–300 × 10^6 spermatozoa/ml was then washed using a 45%/90% Percoll gradient [36, 47]. Next, the sperm pellet was diluted to a concentration of 20 × 10^6 spermatozoa/ml. At least three replicates of each experiment were performed using one ejaculate from each of the three stallions. The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine of Ghent University (EC2013/175 and EC2013/176).

Sperm-Oviduct Binding

Oviduct explants were cultured in DMEM/F12 medium supplemented with 10% FBS, equilibrated with 5% CO_2 in a humidified atmosphere at 38.5°C, as described by Nelis et al. [45]. After overnight incubation, oviduct explants with a diameter of less than 200 μm were selected and washed; five oviduct explants per droplet were then transferred to 45-μl droplets of different media. The binding of sperm to oviduct explants was performed in Whitten medium (100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose, 22 mM Hepes, 2.4 mM sodium lactate pentahydrate, and 1.0 mM pyruvic acid; pH = 7.4 and 280–300 mOsm/kg) at 38.5°C in air, also referred to as noncapacitating medium/conditions. To provide sperm capacitation-supporting conditions, Whitten medium was modified by replacing the sodium lactate pentahydrate with 2.4 mM calcium lactate pentahydrate and adding 25 mM $NaHCO_3$ and 7 mg/ml BSA (pH = 7.4 and 280–300 mOsm/kg; osmolality was adjusted by reducing NaCl); this medium was pre-equilibrated for at least 2 h in a humidified atmosphere containing 5% CO_2 at 38.5°C, and is hereafter referred to as capacitation medium (adapted from McPartlin et al. [48]). In general, a final concentration of 2 × 10^6 spermatozoa/ml was obtained by adding 5 μl Percoll-washed and -diluted sperm (20 × 10^6/ml spermatozoa) to the 45-μl oviduct explant-containing droplet. The droplets were cultured under mineral oil to

prevent evaporation. Three different incubation conditions were applied: noncapacitating conditions (38.5°C in air), DMEM/F12 with 10% FBS, and capacitating medium (38.5°C in a humidified atmosphere equilibrated with 5% CO_2). Each replicate was performed with a different ejaculate.

Oviduct Ciliary Activity and Sperm Binding

Oviduct explant viability was tested in both noncapacitating and capacitating medium at various durations of culture (0, 2, 4, 6, 12, and 24 h). At each time point, viability of oviduct explants was evaluated by assessing ciliary activity using a phase-contrast microscope (magnification of 1000×). The effect of sperm concentration on sperm binding to oviduct explants was assessed in noncapacitating medium using 0.5, 1, 2, 5, or 10×10^6 spermatozoa/ml. After 2 h of coincubation, the sperm-oviduct explants were washed twice in noncapacitating medium and evaluated for sperm-oviduct binding. Subsequently, sperm-oviduct binding was tested in three different coincubation media: DMEM/F12 with 10% FBS (basic culture medium for oviduct explants), noncapacitating medium, and capacitating medium. For each condition, stallion sperm were added to oviduct explants at a concentration of 2×10^6 spermatozoa/ml and incubated for 0.5, 2, 4, or 6 h. In some cases, after a 2-h coincubation in noncapacitating medium, sperm-oviduct explants were washed and transferred to noncapacitating or to capacitating medium for a further 0.5, 3, or 6 h.

Sperm and Oviduct Explant Staining

The number of spermatozoa bound to oviduct epithelium was determined by visualizing the live sperm using 2 μM JC-1 in Hepes-buffered washing medium for 15 min at 37°C in 5% CO_2 in air to stain the mitochondria in the sperm midpiece [49]. This fluorophore can reversibly change its emission from yellow-red (aerobic sperm metabolism) to green (anaerobic metabolism) accompanied by depolarization of the inner mitochondrial membrane [50]. JC-1 was combined with a live/dead cell nucleus stain combination, SYBR14 (20 μM) and PI (50 nM) (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Leiden, The Netherlands) [49]. Stained oviduct explants and sperm-oviduct explant complexes were washed twice in Hepes-buffered washing medium and mounted on siliconized glass slides (Marienfeld, Germany) using 1.4-diazabicyclo[2.2.2] octane (DABCO) as antifade, and sealed with nail polish. Green fluorescence-labeled oviduct cells and sperm heads were considered viable, whereas red oviduct cells and sperm heads were considered dead. Finally, the relative percentages of viable, aerobically metabolizing OECs and spermatozoa were scored by counting the viable cells in five microscopic fields. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400×.

Acrosome Status

The acrosome status of spermatozoa that had been incubated with oviduct epithelial explants for a period of 6 h in noncapacitating and capacitating media was assessed using fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) to discriminate acrosome-intact from acrosome-deteriorated spermatozoa [51]. Briefly, after fixation in 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, sperm-oviduct explants were washed in PBS and further permeabilized by incubating in 0.1% Triton X-100 in PBS for 10 min at room temperature. After washing in PBS, the sperm-oviduct explants were stained for 15 min at room temperature with 1 μg/ml PNA-FITC. After two extra washes with PBS, the stained sperm-oviduct explants were mounted as described above. Spermatozoa with PNA-FITC-labeled acrosome regions were considered acrosome intact, whereas spermatozoa with no fluorescence over the acrosomal region were considered to be acrosome reacted. The relative percentages of acrosome-intact spermatozoa were scored by counting 200 spermatozoa per sperm-oviduct explant incubation. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400×.

Sperm Tail-Associated Protein Tyrosine Phosphorylation

After coincubation, sperm-oviduct explant complexes were washed twice and fixed in 4% paraformaldehyde in PBS at room temperature for 15 min. The fixative was removed by three washing steps using PBS. The washed sperm-oviduct explant complexes were subsequently incubated in 0.1% Triton X-100 in PBS for 10 min at room temperature to ensure full permeabilization of membranes. The immobilized and permeabilized sperm-oviduct explant complexes were then incubated in blocking buffer (PBS containing 1% BSA) for 10 min at room temperature. After this step, spermatozoa were incubated in buffer containing 0.1% BSA and supplemented with a mix of mouse monoclonal 4G10 Platinum IgG_{2b} protein anti-phosphotyrosine antibody (diluted 1:500) and rabbit anti-tubulin antibody (diluted 1:80) at 4°C. After overnight incubation, unbound antibody was removed by gently washing the sperm-oviduct explant complexes twice using 1 ml of PBS containing 0.1% BSA. The resulting sperm-oviduct explant complexes were then stained with a mix of a monoclonal goat anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Molecular Probes, Ghent, Belgium) and a goat anti-rabbit antibody conjugated to Texas red for 1 h at room temperature. After immunolabeling, the two nonbound antibody conjugates were removed by washing three times using PBS containing 0.1% BSA, and once using PBS. The immunolabeled sperm-oviduct explant complexes were mounted on glass slides as described above and sealed with nail polish. The proportion of spermatozoa with green fluorescent tails among the total sperm population (with red fluorescent tails) was determined by counting 200 spermatozoa per sperm-oviduct explant complex. Samples were examined using an Eclipse Ti microscope (Nikon, Tokyo, Japan) equipped with a mercury lamp and appropriate filters, at a minimum magnification of 400×.

Assessing pH_i of OECs and Spermatozoa

Oviduct explants and sperm-oviduct explant complexes were washed twice using Hanks Balanced Salt Solution (HBSS) and subsequently stained with 5 μM of the pH-sensitive dye BCECF-AM. After a 30-min incubation at 38.5°C, the noncellular dye was removed by washing the complexes twice in HBSS. The complexes were then resuspended in medium and incubated for an additional 20 min to allow de-esterification of the dyes, and mounted on glass slides using DABCO as antifading agent and to help immobilize spermatozoa for assessing pH_i. To determine the pH_i of sperm cells, a calibration was first performed using BCECF-AM-equilibrated spermatozoa in the presence of 0.1% Triton X-100 and by adjusting the pH with HCl and NaOH [52, 53]. Increasing relative green fluorescence was related to increasing pH_i [54]. The BCECF signal was measured in OECs and during 6-h sperm-oviduct explant coincubations in bound as well as unbound spermatozoa. Samples were examined using a Leica DMR microscope equipped with a Hg-lamp and appropriate filters, at a magnification of 400x and 1000×.

Sperm Motility Assessment

Motility patterns during sperm-oviduct explant binding and after sperm release from the oviduct explants were assessed using a CCD ICD-46E camera (Ikegami Tsushinki Co. Ltd., Japan) attached to an Olympus IX70 inverted microscope (Olympus Belgium N.V., Aartselaar, Belgium). Images were acquired using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium).

Microscopic Imaging of Sperm-Oviduct Binding

Density of spermatozoa bound to the oviduct explants, along with membrane integrity, acrosome status, and pH_i of bound spermatozoa during the 6-h coincubation were determined in five microscopic fields (400× magnification) by means of fluorescence microscopy using a Leica DMR microscope equipped with excitation filters BP 340/380 nm, BP 450/490 nm, BP 560/40 nm, and a 100-W mercury lamp. Alexa Fluor 488-conjugated goat anti-mouse antibody, SYBR14, PNA-FITC, PI, Texas red-conjugated goat anti-rabbit antibody, JC-1, Hoechst 33342, and BCECF-AM ester were sequentially excited using 499-, 498-, 495-, 536-, 589-, 592-, 345-, and 490-nm wavelength and subsequently showed emission spectra of 519-, 522-, 519-, 617-, 615-, 595-, 478-, and 530-nm wavelength. These emission spectra were detected by blue (LP 425 nm), green (LP 515 nm), and red (BP 645/75 nm) filters corresponding to the emission peaks of the dyes. Images were acquired using the Image Database program (Leica, Van Hopplynus N.V.). In addition, the surface area of the oviduct explants in each microscopic field (at a magnification of 400×) was measured using this program. The percentage of spermatozoa with tail-associated protein tyrosine phosphorylation (PY^+/TUB^+) was determined by evaluating 200 randomly selected spermatozoa (TUB^+); the acrosome status of bound spermatozoa ($PNA\text{-}FITC^+/TUB^+$) at each time point of coincubation was similarly evaluated by confocal microscopy using a Bio-Rad Radiance 2100 MP system (Zeiss/Bio-Rad, Hertfordshire, U.K.) attached to a Nikon Eclipse TE300 inverted microscope (Nikon, Badhoevedorp, The Netherlands) at a magnification of 400× or 1000×. The fluorescent dyes were excited using an argon laser and analyzed using the same filters as described for fluorescence microscopy. Images were acquired using LaserSharp 2000 software (Zeiss/Bio-Rad) after background corrections. For each wavelength, digital optical sections were collected using Z-series acquisition at intervals of 0.35 μm. In both systems, the various fluorophores were checked for signal overlap; no leakage of signals was detected.

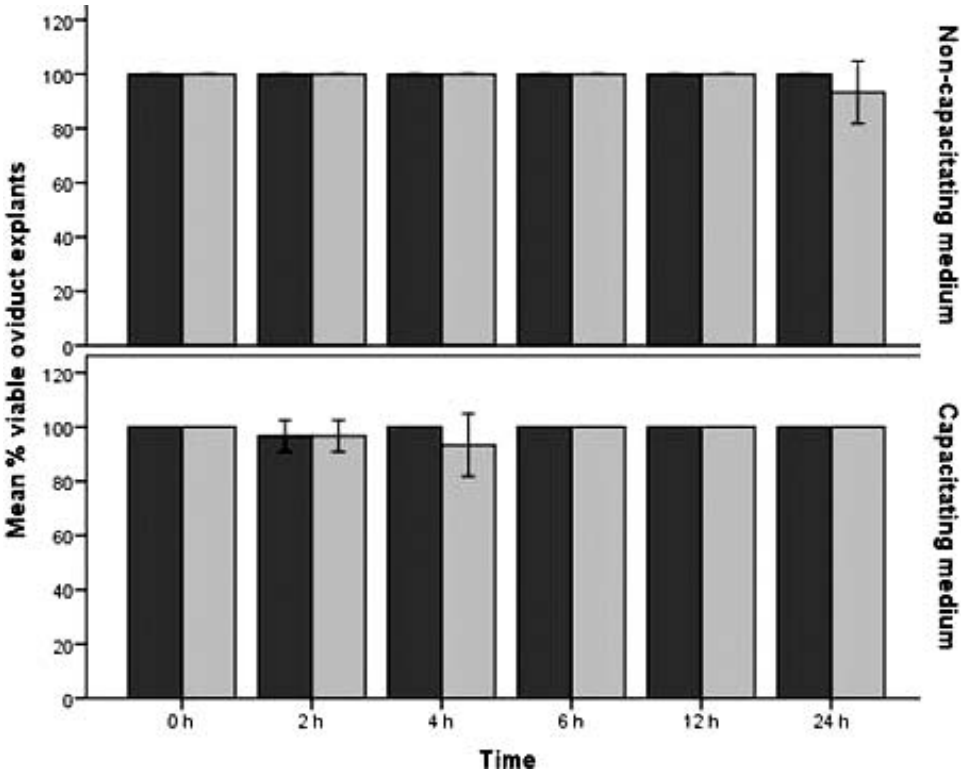

FIG. 1. Oviduct explant viability during 24-h incubation in noncapacitating and capacitating media. Data represent mean (±SD) percent of oviduct explants (n = 30/group) showing ciliary activity (dark gray bars) and membrane integrity (light gray bars) over three replicates. ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.

Statistical Analysis

The effects of treatments on sperm parameters were assessed by ANOVA using the general linear model procedure of SPSS version 20 for Windows (SPSS IBM, Brussels, Belgium). The number of spermatozoa bound to oviduct explants and the percentages of PI^- and $SYBR^+$ spermatozoa, as well as the change in the percentage of tyrosine-phosphorylated (PY^+) spermatozoa, over time were calculated as repeated measures with Greenhouse-Geisser correction by the general linear model and a Bonferroni correction. Post hoc tests were performed by Sheffé analysis. Differences were considered significant at $P < 0.05$.

RESULTS

Viability of Oviductal Explants

We previously demonstrated that oviduct explants remained viable for up to 6 days when incubated in DMEM/F12 medium with 10% FBS [45]. In order to assess aspects of sperm-oviduct binding, however, the possibility that the relatively simple sperm incubation media may compromise oviduct explant viability was examined by assessing changes in morphological features (ciliary activity and membrane integrity) of explants incubated for 24 h in noncapacitating and capacitating media. We were able to demonstrate that oviduct explants remained viable for at least 24 h when incubated in these media (Fig. 1); indeed, the vast majority of oviduct explants displayed ciliary activity that was very similar between media and changed little over time (99% ± 2%; $P = 0.44$; Fig. 2a and Supplemental Video S1; all Supplemental Data are available online at www.biolreprod.org), while all cells (100% ± 0%; $P = 0.67$; Fig. 2b) of nearly all explants (>99%) remained membrane intact.

Sperm-Binding Capacity of Equine Oviduct Explants

To standardize the sperm-oviduct binding assay, the saturation concentration for sperm binding to oviduct explants was evaluated by exposing oviduct explants to increasing sperm concentrations (0.5, 1, 2, 5, and 10×10^6 spermatozoa/ml) in noncapacitating medium. Saturation for sperm binding was reached at 5×10^6 spermatozoa/ml ($1.9 \pm 0.4 \times 10^5$ spermatozoa/mm^2; see Fig. 3).

At different time points (0.5, 2, 4, and 6 h), sperm-oviduct binding was quantified by using a sperm concentration of 2×10^6 spermatozoa/ml in 45-μl droplets of noncapacitating medium and compared to both capacitating medium and the previously described DMEM/F12 with 10% FBS. The mean ± SD number of bound spermatozoa at different time points (0.5, 2, 4, and 6 h) was $10.0 \pm 2.7 \times 10^5$ spermatozoa/mm^2 in noncapacitating medium compared to $6.0 \pm 1.1 \times 10^5$ spermatozoa/mm^2 in capacitating medium and $1.4 \pm 0.4 \times 10^5$ spermatozoa/mm^2 in DMEM/F12-based culture medium; these sperm-binding densities differed significantly between media ($P < 0.001$). Within a medium, there was no significant effect of incubation time on the number of bound spermatozoa (DMEM/F12-based culture medium, $P = 0.25$; noncapacitating medium, $P = 0.07$; capacitating medium, $P = 0.80$; Fig. 4).

Selective Binding of Intact Sperm to Oviduct Explants

Plasma membrane and acrosome integrity of sperm bound to oviduct explants were assessed during 6-h coincubation and compared to those of the unbound sperm fraction. It transpired that membrane-intact spermatozoa were more prevalent among the oviduct-bound sperm population (99.0% ± 0.5%) compared to the unbound sperm fraction (80% ± 2.5%) ($P < 0.001$; Figs. 5 and 6, a and b). The same was true for acrosome

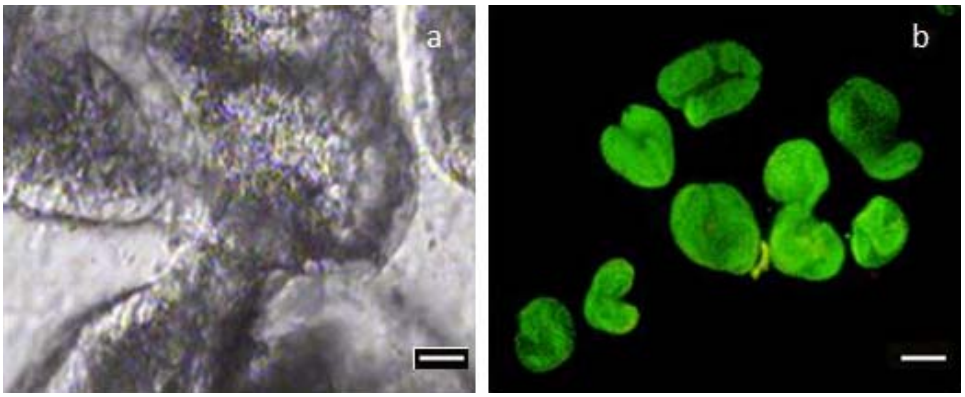

FIG. 2. **a**) Representative image from Supplemental Video S1 of oviduct explants after 24-h incubation in noncapacitating Whitten medium or capacitating modified Whitten medium. In 99% of the oviduct explants, ciliary activity was evident after 24-h incubation. **b**) Fluorescence microscopic image of oviduct explants after 24-h incubation in noncapacitating Whitten medium or capacitating modified Whitten medium. SYBR14/PI staining demonstrated that more than 99% of the cells were membrane intact after 24-h incubation (green, membrane intact). **a**) Original magnification ×1000; bar = 62.5 μm. **b**) Original magnification ×100; bar = 6.25 μm.

integrity (99.6% ± 0.8% for oviduct explant bound spermatozoa versus 72% ± 11% for nonbound spermatozoa; Figs. 5 and 6, c and d); again, these differences ($P < 0.001$) were not time dependent ($P = 0.63$), but were already apparent at time point 0.5 h.

Sperm-Oviduct Binding Induced Protein Tyrosine Phosphorylation in Sperm Tails

The hypothesis that in vivo sperm capacitation is initiated during residence in the sperm reservoir [2] led us to investigate the effect of sperm-oviduct binding on tail-associated protein tyrosine phosphorylation, which is thought to be an essential step in capacitation. The percentage of membrane-intact spermatozoa with tail-associated protein tyrosine phosphorylation was significantly higher for the oviduct explant-bound sperm fraction than among the unbound population ($P <$ 0.001). After 6-h coincubation, the percentage of protein tyrosine-phosphorylated sperm increased to 43% ± 5% of the bound sperm population in noncapacitating medium compared to 60% ± 16% in capacitating medium (Figs. 5 and 7, a–c). By comparison, only a small percentage of unbound sperm in the same medium showed evidence of protein tyrosine phosphorylation after 6 h (6% ± 2% and 12% ± 1%, respectively).

Alkaline Secretory Activity of Oviduct Explants and the Effects on Spermatozoa pH

It has been reported that equine spermatozoa become protein tyrosine-phosphorylated after incubation in medium with an external pH of 8, achieved by incubation in air [40, 41].

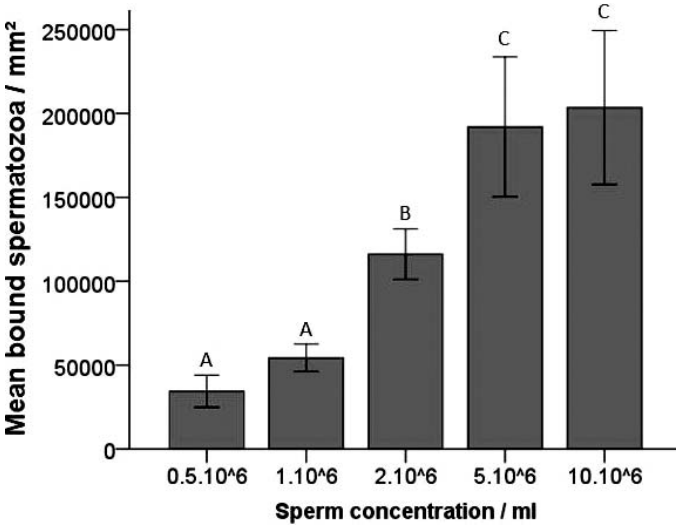

FIG. 3. Effect of sperm concentration on binding capacity of equine oviduct explants for stallion sperm under noncapacitating conditions. Saturation of sperm-oviduct explant binding was detected at 5×10^6 spermatozoa/ml. Data represent mean (±SD) number of spermatozoa bound to oviduct explants (n = 30/group) in four replicates. Values that differ significantly are indicated by different capital letters. ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.

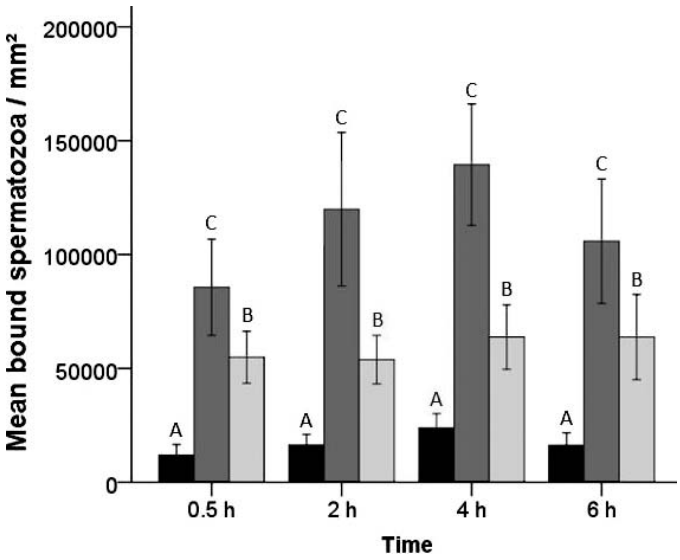

FIG. 4. Effect of media on sperm binding capacity of oviduct explants over time. Data represent mean (±SD) number of spermatozoa bound to oviduct explants (n = 10/group) over four replicates. Noncapacitating medium (dark gray bars) supported sperm-oviduct binding at a higher level than capacitating medium (light gray bars). DMEM/F12 basic culture medium supplemented with 10% FBS (black bars) supported the lowest sperm-oviduct binding. Within each time point, values that differ significantly are indicated by different capital letters. Repeated measures ANOVA with Greenhouse-Heisser correction; post hoc tests with Bonferroni correction was performed.

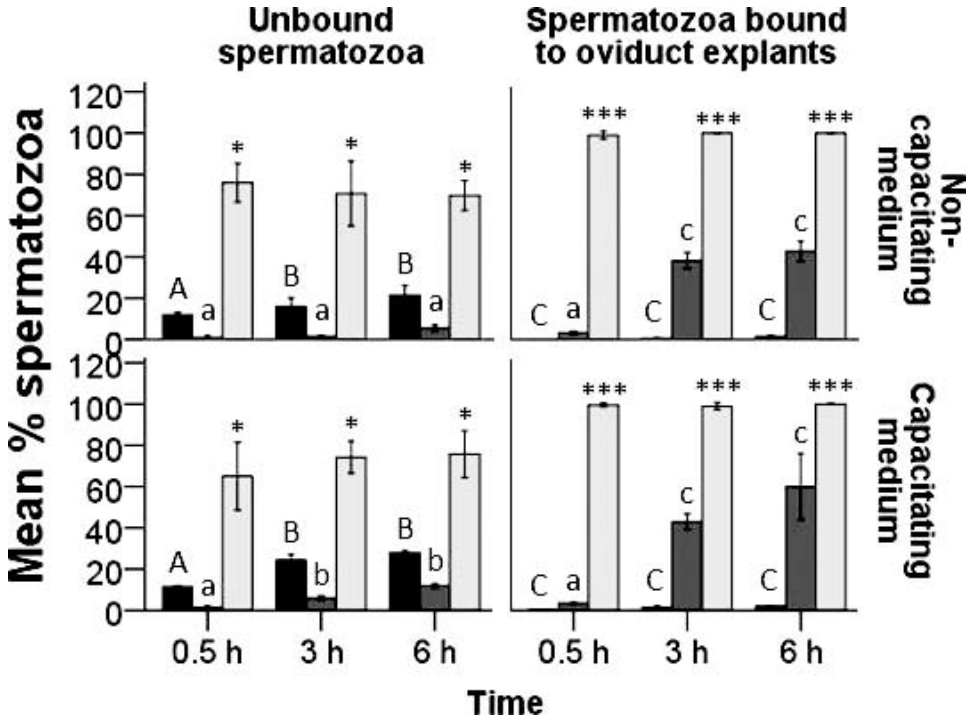

FIG. 5. Percentage of plasma membrane-intact, protein tyrosine-phosphorylated and membrane-intact, or acrosome-intact spermatozoa in noncapacitating and capacitating media over time, for sperm in suspension or for sperm bound to oviduct explants. In the oviduct explant-bound sperm population, a time-dependent increase in tail-associated protein tyrosine phosphorylation was observed in both conditions. Plasma membrane and acrosome integrity were conserved during sperm-oviduct binding. Data represent mean (±SD) percentages of membrane-damaged (black bars), membrane-intact and protein tyrosine-phosphorylated (dark gray bars), and membrane-intact and acrosome-intact spermatozoa (light gray bars) (n = 10 oviduct explants in each group; three replicates). For the percentage of membrane-damaged spermatozoa, values that differ significantly are indicated by different capital letters. For the percentage of membrane-intact and protein tyrosine-phosphorylated spermatozoa, values that differ significantly are indicated by different lowercase letters. For the percentage of membrane-intact plus acrosome-intact spermatozoa, values that differ significantly are indicated by asterisks. Repeated measures ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.

In order to explain the increased protein tyrosine phosphorylation observed in oviduct-bound spermatozoa, we investigated whether this interaction induces alkalization of spermatozoa. BCECF-AM staining was performed on oviduct explants and oviduct-bound spermatozoa, and pH_i of both types of cells was assessed over time. Oviduct explants recovered from mares at the late-preovulatory stage of the cycle contained secretory cells with a mildly alkaline apical segment (pH = 7.5–7.8; Fig. 8, i and j). As incubation progressed, the incidence of oviductal cells with an alkaline content decreased (121 ± 23, 112 ± 25, 76 ± 19, and 23 ± 11 cells/mm^2 at 0.5, 1, 3, and 6 h, respectively). By contrast, the pH_i of spermatozoa bound to the oviduct cells increased over time (6.82 ± 0.12, 7.14 ± 0.30, 7.70 ± 0.16, and 7.68 ± 0.12 at 0.5, 1, 3, and 6 h, respectively; Fig. 8, k–n). Unbound sperm also exhibited a significant pH_i rise over time (6.79 ± 0.57, 6.86 ± 0.84, 7.02 ± 0.14, and 7.17 ± 0.12 at 0.5, 1, 3, and 6 h, respectively), but the pH values were consistently lower than for oviduct explant-bound spermatozoa ($P < 0.001$). Comparing the timing of intracellular alkalization and protein tyrosine phosphorylation in oviduct explant-bound sperm indicated that alkalization preceded protein tyrosine phosphorylation (Fig. 5 versus Fig. 9; $P < 0.01$).

DISCUSSION

In vivo, mammalian spermatozoa follow an ordered sequence of events in preparation for the fertilization of an oocyte [55]. For equids, the proposed sequence of events includes: 1) stallion spermatozoa are ejaculated into the uterine body and transported to the utero-tubal junction (UTJ); 2) a reservoir of noncapacitated spermatozoa is established at the UTJ; 3) spermatozoa within the reservoir become capacitated as the time of ovulation approaches; 4) the capacitated spermatozoa are released from the sperm reservoir having acquired hyperactivated motility; 5) the released spermatozoa meet the mature oocyte at the ampullary-isthmic junction; 6) after recognition, the spermatozoa bind to the zona pellucida or the intercellular matrix of the cumulus cell complex [56, 57]; 7) the acrosome reaction is subsequently triggered; 8) this reaction allows the sperm to penetrate the cumulus and zona pellucida and enter the perivitelline space; and 9) from there, the fertilizing spermatozoon can bind and fuse with the oolemma. The effect of the oviduct on equine sperm physiology is the subject of the current study.

Conventional IVF with equine gametes is not successful [32–34], whereas oocyte transfer [58] and ICSI [31, 59, 60] can be applied successfully in horses. This implies that stallion spermatozoa are able to fulfill their role in fertilization in vivo or after injection into an oocyte. In contrast to most other mammalian species, stallion spermatozoa are not efficiently activated by conventional IVF or in vitro capacitation media (i.e., media containing bicarbonate, Ca^{2+}, and defatted albumin as capacitation-supporting factors, with or without an additional PKA inducer). In vivo, the oviduct almost certainly plays a critical role in regulating sperm activation, and it is tempting to speculate that oviduct-sperm interactions are essential to eliciting capacitation of stallion spermatozoa. Of course, it is difficult to monitor sperm-oviduct interactions in situ. Therefore, in the current study, an ex vivo equine sperm-

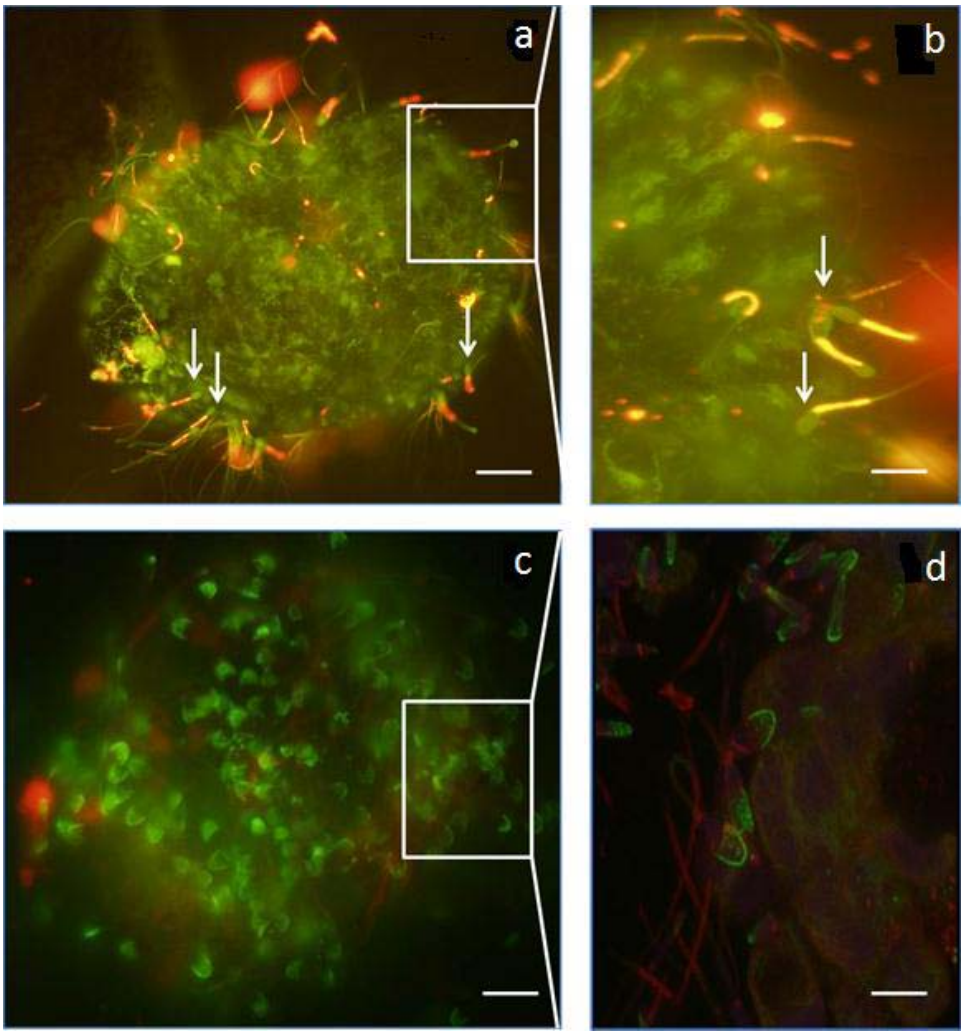

FIG. 6. **a** and **b**) Fluorescence micrographs of spermatozoa bound to oviduct explants. As demonstrated by SYBR14/PI/JC-1 staining, the bound spermatozoa remained membrane-intact (green) for at least 6 h in noncapacitating and capacitating media (white arrows). **c**) Acrosome integrity of spermatozoa bound to oviduct explants after 6-h coincubation in noncapacitating and capacitating media evaluated by PNA-FITC using fluorescence and (**d**) confocal microscopy. **a** and **c**) Original magnification ×400; bar = 25 μm. **b** and **d**) Original magnification ×1000; bar = 62.5 μm.

oviduct binding model was used to mimic the events that take place in the oviduct during the late preovulatory stage.

Equine oviductal explants (in which morphological and ultrastructural features, such as ciliary beating, are well preserved [45]) were used. This system was selected primarily because ciliation in bovine oviductal epithelial cells is thought to be a terminal differentiation event that is difficult to induce or maintain in vitro [61]. Moreover, because bull [46] and boar [62] sperm bind preferentially to the cilia or to deeper regions of ciliated, and not to the secretory, epithelial cells; oviduct epithelial explants are preferred to less differentiated oviduct monolayers. Baillie et al. [63] reported that, in humans, spermatozoa bind more avidly to explants than monolayers. We did not investigate this preference further in the current study, but found the oviduct explant system to be very practical, because, as for cattle [64], the explants could be used within 6–12 h of harvesting, whereas monolayers can only be used several days later [45].

To establish the in vivo-like equine oviduct explant culture, epithelial cells were recovered from the ampullary-isthmic junction. In vivo, on the other hand, the sperm reservoir is not thought to extend beyond the first few centimeters of the isthmus proximal to the UTJ. However, it is technically almost impossible to reliably and repeatedly collect OECs exclusively from the equine isthmus, because of its very narrow lumen and tortuous anatomy [6, 65]. Moreover, because sperm-OEC binding characteristics have been shown to be independent of the oviductal site of origin in cattle [7, 11], we decided to harvest cells from the isthmic-ampullary junction where we could at least reliably harvest a comparable cell population (i.e., from the same location) for each replicate. While it is also likely that sperm in the oviductal reservoir are exposed to ampullary secretions in vivo, we do acknowledge that the absence of regional differences in OEC binding properties has not been proven for the horse. In this respect, it remains possible that cells from the distal isthmus may display different sperm-oviduct interactions from those at the ampullary-isthmic junction, which would affect interpretation of the outcome of the binding studies.

With regard to the quantification of sperm-oviduct binding, we were aware that standardization is the main challenge when using equine oviduct explants, due to the invaginated and irregular oviductal surface, which is very different from the flattened surface of an oviduct monolayer [66, 67]. To achieve standardization in the oviduct explant model, we decided that the ideal sperm concentration needed to be below the sperm-

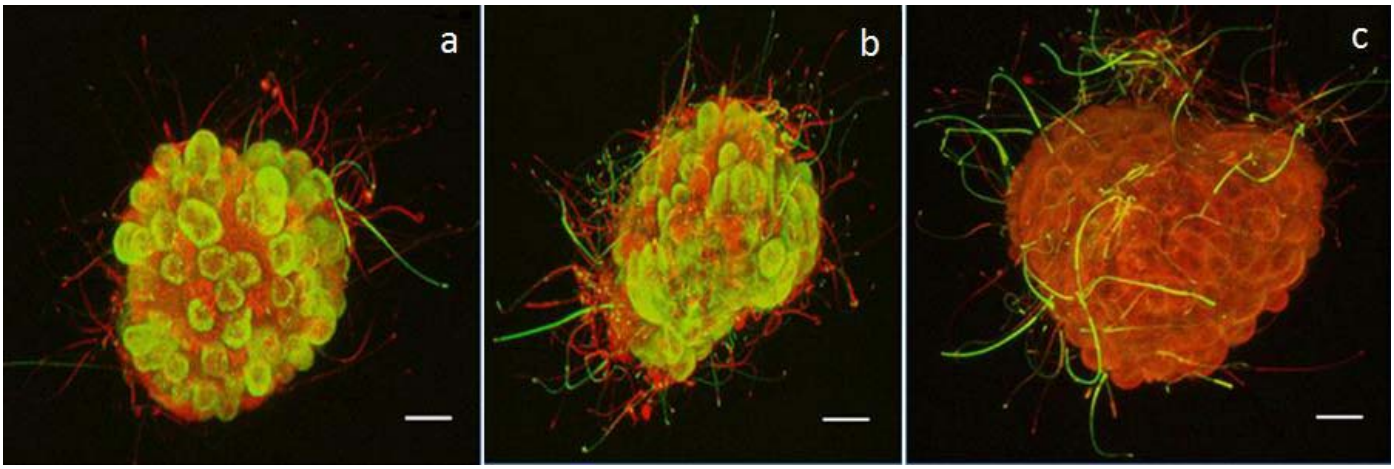

FIG. 7. Fluorescence micrographs of protein tyrosine-phosphorylated spermatozoa bound to oviduct explants after 6-h coincubation in capacitating medium. Bound spermatozoa were identified by means of confocal microscopy after double indirect immunofluorescence staining (red, tubulin; green, protein tyrosine phosphorylation) at 30 min (**a**), 3 h (**b**), and 6 h (**c**) of coincubation in capacitating medium. Tyrosine phosphorylation increased over time. **a**–**c**) Original magnification ×400; bar = 25 μm.

oviduct saturation level (5×10^6 spermatozoa/ml), and this was set in noncapacitating conditions at 2×10^6 spermatozoa/ml.

Interestingly, the oviduct explants were able to selectively bind intact spermatozoa, which is in agreement with previous observations made in pigs [9] and cattle [10], where sperm-oviduct binding was shown to be a useful method for selecting and preserving plasma membrane and acrosome-intact spermatozoa. We confirmed that only membrane- and acrosome-intact spermatozoa bound to oviduct explants, and that they maintained their intact status for at least 6 h. Oviduct explant binding was most efficient in incubation media devoid of capacitation-supporting factors, in which sperm binding capacity was approximately twice that of incubation in capacitation-supporting media. Similar results have been reported in cattle [44] and hamsters [68], and support the hypothesis of Suarez [1] that only noncapacitated spermatozoa

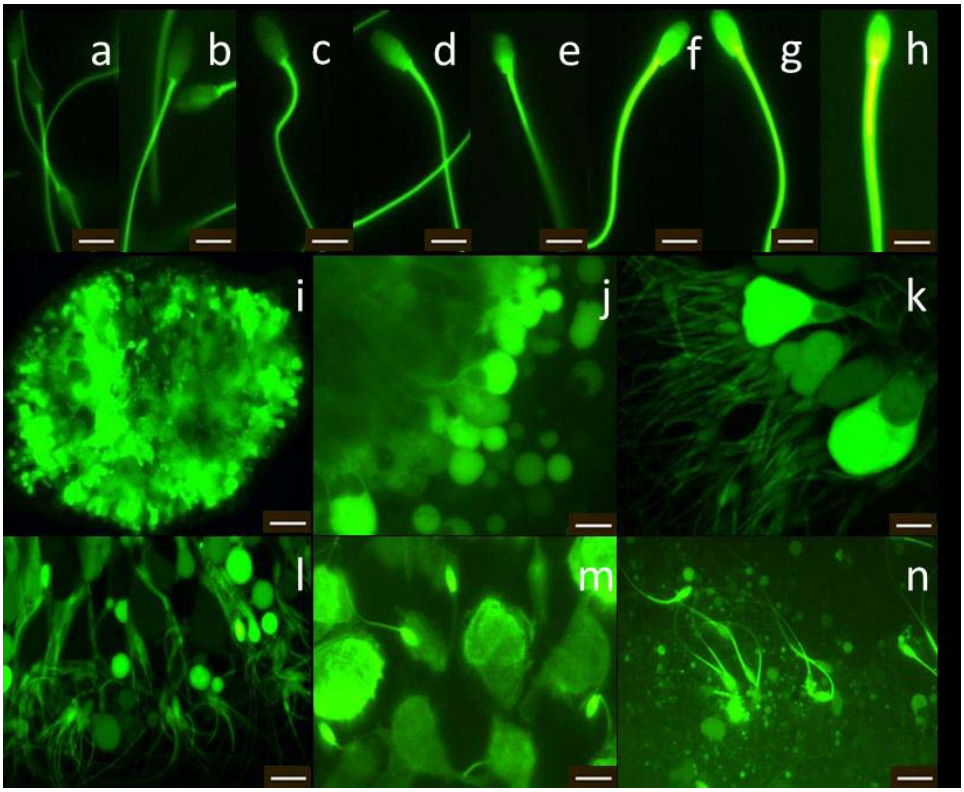

FIG. 8. Fluorometric recordings of pH_i of (**a**–**h**) pH-calibrated spermatozoa (pH 6.7, 6.8, 6.9, 7.1, 7.3, 7.5, 7.9, and 8.3), (**i** and **j**) oviduct explants, and (**k**–**n**) oviduct-bound spermatozoa after 0.5, 1, 3, and 6 h. As demonstrated by BCECF-AM staining, secretory epithelial cells of oviduct explants contained intracellular content with increased pH. During 6-h sperm-oviduct explant binding, the pH_i of bound spermatozoa reached a maximum at 3 h, which was maintained until 6 h. Additionally, the alkaline secretory content of epithelia was released gradually during the 6 h of sperm-oviduct binding. Images were recorded using fluorescence microscopy. **i**) Original magnification ×400; bar = 25 μm. **a**–**h** and **j**–**n**) Original magnification ×1000; bar = 62.5 μm.

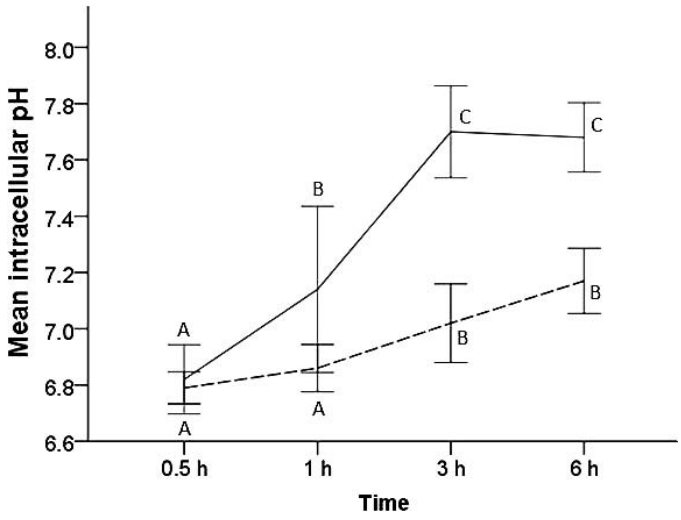

FIG. 9. Mean pH_i recorded in oviduct-bound spermatozoa (black solid line) and sperm suspensions (black dotted line) over time. In both sperm populations, a time-dependent increase in pH_i was observed. However, the pH_i of oviduct-bound spermatozoa tended to alkalize after 3-h sperm-oviduct binding, whereas this did not occur in sperm in suspension. Data represent mean (±SD) pH_i per spermatozoa (n = 50/group) over three replicates. Within the oviduct-bound spermatozoa and suspended sperm populations, values that differ significantly are indicated by different capital letters. Repeated measures ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.

are able to bind to oviduct epithelium during estrus. The reduced sperm binding capacity observed under capacitating conditions may be a factor in a substantial population of spermatozoa undergoing membrane changes that decrease their affinity for oviduct epithelia. Sperm plasma membrane changes are an early and integral part of the stallion sperm capacitation process [69], and are precursors to critical intracellular changes, such as PKA activation and subsequent protein tyrosine phosphorylation. We showed that incubation of spermatozoa in capacitation-supporting conditions (HCO_3^-, BSA, and Ca^{2+}) was a poor stimulator of protein tyrosine phosphorylation, whereas binding of spermatozoa to oviduct explants caused a spectacular increase in the percentage of sperm displaying protein tyrosine phosphorylation. On the other hand, significant release of spermatozoa from oviduct epithelia was not observed in any of the media tested. The fact that spermatozoa showed the lowest binding in the DMEM/F12-based oviduct explant culture medium that contained 10% FBS may be explained by the presence of many blocking proteins in FBS that could conceivably saturate oviduct binding receptors [70, 71].

One of the central changes triggered during sperm capacitation is the generation of hyperactivated sperm motility, which, in boar sperm, is a process induced by massive protein tyrosine phosphorylation [23]. Only a small proportion of the spermatozoa suspended in in vitro capacitation media showed a visible protein tyrosine phosphorylation response. However, in both the absence and, in particular, the presence of capacitation-supporting factors, binding to preovulatory oviduct explants induced protein tyrosine phosphorylation in approximately half of the spermatozoa, although hyperactivated motility was not observed. Moreover, the nonbound sperm showed no such response, indicating that prolonged direct contact with the oviduct epithelium is required for the protein tyrosine phosphorylation response. Sperm-oviduct binding is regarded in many species (e.g., pigs [9] and cattle [10]) as a mechanism to select membrane-intact, acrosome-intact, and noncapacitated spermatozoa. During the 2-h coincubation of spermatozoa with oviduct explants, a gradual increase in oviduct binding was evident. After two washing steps, established sperm-oviduct explants were transferred to either noncapacitating or capacitating medium. In both conditions, only minimal release of bound spermatozoa was observed during the subsequent 6-h incubation. Possibly, unknown in vivo factors (such as the influx of follicular fluid postovulation, chemoattractant components derived from the cumulus oocyte complex entering the oviduct, or simply the stream of fluids through the oviduct) may be required for sperm release. In any case, the protein tyrosine phosphorylation induced in vitro by sperm binding to oviduct explants was insufficient to allow sperm release from the oviduct cells. This also suggests that the unbound fraction almost certainly did not first bind to the oviduct epithelium and then detach soon after, but within the 2-h coincubation period. This conclusion is supported by the following observations. First, in the unbound sperm population, a minimal percentage of spermatozoa showed protein tyrosine phosphorylation. If this population had previously bound, we assume that the rates of protein tyrosine phosphorylation would have been higher. Second, stallion sperm incubated in capacitation media in the absence of oviduct epithelium showed similar minimal protein tyrosine phosphorylation response rates (<10% after 6 h; data not shown).

Interestingly, a similar protein tyrosine phosphorylation response has been reported for stallion sperm suspensions when the pH of the capacitation medium was alkaline [40, 41]. It may, therefore, be significant that the secretory cells in the oviduct explants contained large alkaline vesicles, although these only marginally raised the pH of the incubation medium. In vivo, however, the alkaline secretions may be diluted to a much lesser extent by oviduct fluid and may, therefore, induce an alkaline local microenvironment that is sufficient to induce sperm protein tyrosine phosphorylation without the need for the sperm to bind to the oviduct. Certainly, in our in vitro oviduct explant system, direct contact between spermatozoa and oviduct explants was necessary. Indeed, the pH of the adhered spermatozoa reached levels similar to those described in alkalinized capacitation media [40]. The direct contact of stallion spermatozoa to the oviduct epithelium was thus sufficient to induce intracellular alkalization consistent with capacitation induction. In the female reproductive tract, the pH has been reported to exceed 7, reaching values of up to pH 8.0 in cervical mucus and pH 7.4 in the equine oviduct [72], although oviductal pH is also affected by the stage of the estrous cycle [72]. Combining the various observations, it is hypothesized that a local increase in the HCO_3^- concentration is essential for stallion sperm to capacitate and subsequently fertilize [73].

During sperm transport through the female reproductive tract, the pH_i of a murine or human spermatozoon increases, accompanied by an additional decrease in extracellular H^+ concentration, and leading to a slightly alkaline pH, which is consistently lower than the extracellular pH, due to the presence of transmembrane ion pumps [74]. Our finding supports and extends previous studies describing the necessity of an alkaline environment for stallion sperm capacitation, at the level of protein tyrosine phosphorylation. The novel aspect of this study is the finding that this process can be elicited by binding to preovulatory oviduct explants, which makes it likely that an analogous process takes place in vivo. Further research should focus on how the OEC-sperm contact results in induction of protein tyrosine phosphorylation.

In contrast to the present study, it has been shown that horse sperm binding to OEC monolayers reduces Ca^{2+} metabolism, leading to a temporary decrease in motility, inhibition of capacitation, and increased sperm survival time [75, 76]. Aside from a low intracellular Ca^{2+} concentration, Kirichok and Lishko [74] reported a high intracellular H^{+} concentration during this phase, which they proposed to have a complementary effect on mammalian sperm survival. To date, it is clear that stallion spermatozoa can remain viable in the mare's sperm reservoir for at least 6 days [72]. In contrast to oviduct explants, monolayers do not typically retain their morphological and physiological characteristics during culture. In particular, the number of secretory cells decreases dramatically, such that the release of molecules from secretory granules is likely to be marginal when monolayers are used, whereas it is much better conserved in equine oviduct explants [45]. Due to this maintenance of secretory activity, the equine oviduct explant model almost certainly mimics in vivo conditions better than an epithelial cell monolayer [45]. Moreover, the loss of the alkaline secretory vesicles in cells cultured in monolayers, but their retention in oviduct explant vesicles, probably explains the divergence in their effects on the physiology of bound sperm.

Our findings suggest that physical sperm-oviduct epithelium contact during the late-preovulatory period is important to switch on intracellular processes involved in sperm capacitation. This enhancement of capacitation may involve interaction with oviduct plasma membrane molecules or with capacitating factors derived from the secretory OECs. As demonstrated by Suarez and Pacey [2], species-specific carbohydrate moieties expressed on the epithelium are responsible for oviduct epithelium binding to the sperm head. This specific cell contact may prepare spermatozoa for capacitation, where the importance of physical contact is underlined by the failure of oviduct explant-conditioned medium to stimulate enhanced sperm tail protein tyrosine phosphorylation. This rules out the possibility that any procapacitation factors are simply released by the secretory epithelial cells of preovulatory oviduct and are active in the surrounding milieu. We therefore hypothesize that a rise in alkaline secretory activity takes place in the mare's oviduct at the late follicular stage and that physical interaction of stallion spermatozoa with OECs induces alkalization and the first stages of capacitation (membrane changes and tail-associated protein tyrosine phosphorylation).

One note of caution is that, despite the alkalization and protein tyrosine phosphorylation responses of stallion spermatozoa bound to the oviductal explants, there was little or no spontaneous release of activated spermatozoa. In comparison to pigs [23], our study did not show a correlation between protein tyrosine phosphorylation and sperm release by hyperactivation. This release is a prerequisite for fertilization; to date, only sperm incubation with procaine has been shown to reliably induce hyperactivated motility in equine sperm [77, 78].

In conclusion, we showed that oviduct explants harvested during the preovulatory period selectively bound membrane-intact sperm and induced protein tyrosine phosphorylation, probably by increasing pH_i via direct sperm-oviduct epithelial contact. Other capacitation tests (membrane lipid order, Ca assay, acrosome integrity, and oocyte penetration) and subsequent demonstration of the triggering of the release from oviduct epithelium of spermatozoa exhibiting hyperactivated motility would greatly complement this study.

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