Steroid hormones regulate sperm–oviduct interactions in the bovine

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Abstract

After insemination in the cow, a sperm reservoir is formed within the oviducts, allowing the storage and then progressive release of spermatozoa toward the ovulated oocyte. In order to investigate the hormonal regulation of these events *in vitro*, the ovarian steroids 17 β -estradiol (E2) and progesterone (P4) were added at various concentrations to monolayers of bovine oviduct epithelial cells (BOEC) before or during co-incubation with spermatozoa. Main findings demonstrate that (1) a 18-h pretreatment of BOEC with 100 pg/mL and 100 ng/mL of E2 decreased by 25% the ability of BOEC to bind spermatozoa after 10 min, and for the highest dose of E2, 60 min of co-incubation; (2) P4 at concentrations of 10, 100 and 1000 ng/mL induced the release within 60 min of 32–47% of bound spermatozoa from BOEC; this sperm-releasing effect was maintained after a 18-h pretreatment of BOEC with 100 pg/mL of E2; (3) E2 in concentrations above 100 pg/mL inhibited the releasing effect of P4 on bound sperm in a dose-dependent manner; (4) spermatozoa bound to BOEC, then released from BOEC by the action of P4-induced higher cleavage and blastocyst rates after *in vitro* fertilization than the control group. These results support the hypothesis that the dynamic changes in steroid hormones around the time of ovulation regulate the formation of the sperm reservoir and the timed delivery of capacitated spermatozoa to the site of fertilization.

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Introduction

After mating or insemination in mammals, only a limited number of spermatozoa enter the oviducts, where many of them soon bind to the oviductal epithelium in the so-called ‘functional sperm reservoir’ (Suarez 2016). In the bovine, the sperm reservoir is formed over a period of 8–12 h after insemination and spermatozoa can be stored for hours up to days before they are assumed to be released as capacitated cells and move toward the site of fertilization around the time of ovulation (Hunter & Wilmut 1984, Hung & Suarez 2010).

These interactions between oviduct epithelial cells (OEC) and spermatozoa are believed to play an important role in sperm selection in a number of species including the bovine. Only spermatozoa with intact plasma membrane and acrosome (Thomas *et al.* 1994, Lefebvre & Suarez 1996, Gualtieri & Talevi 2000), superior morphology and normal chromatin structure (Ellington *et al.* 1999a) can bind to OEC *in vitro*. The attachment to OEC was found to have beneficial effects on sperm motility, survival and fertilizing ability

(Ellington *et al.* 1991, Pollard *et al.* 1991, Gualtieri & Talevi 2000, 2003). Furthermore, the ability of sperm to bind to OEC *in vitro* appears to be correlated with *in vivo* fertility in bulls (De pauw *et al.* 2002), stallions (Ellington *et al.* 1999b) and pigs (Waberski *et al.* 2005).

A synchronized sperm binding followed by the release from the oviductal reservoir is necessary to allow capacitated spermatozoa to be present around the oocyte at the time of ovulation. The release of bovine spermatozoa from OEC monolayers can be induced *in vitro* by heparin, sulfated heparin-like glycoconjugates and the endocannabinoid anandamide (Talevi & Gualtieri 2001, 2010, Gervasi *et al.* 2009). However, very little is known about the regulation of these events *in vivo*. The formation of the sperm reservoir occurs at the end of the follicular phase of the estrous cycle in parallel with high concentrations of 17 β -estradiol (E2) and low concentrations of progesterone (P4) in the circulating plasma (Glencross *et al.* 1973) and locally within the oviduct (Lamy *et al.* 2016). By contrast, the release of spermatozoa from the sperm reservoir occurs around the time of ovulation, in parallel with decreasing

concentrations of E2 and increasing concentrations of P4 (Glencross *et al.* 1973, Lamy *et al.* 2016). Stimulating roles of P4 at various doses were found in mammalian sperm capacitation, hyperactivated motility, acrosome reaction and chemical guidance (Baldi *et al.* 2009, Guidobaldi *et al.* 2012, Fujinoki *et al.* 2016) while E2 was reported to inhibit P4-induced acrosome reaction (Vigil *et al.* 2008, Baldi *et al.* 2009) and sperm hyperactivation (Fujinoki 2010). However, the potential roles played by the periovulatory dynamic changes in P4 and E2 in the regulation of sperm–oviduct interactions are poorly known.

In the porcine, the binding of spermatozoa to oviductal explants *in vitro* was not affected by the stage of the estrous cycle but was increased by addition of 70 pg/mL of E2 to the co-culture medium (Suarez *et al.* 1991). On the other hand, the pretreatment of porcine oviduct explants with P4 but not E2 modulated the ability of epithelial cells to bind spermatozoa (Bureau *et al.* 2002). Moreover, the experimental microinjection of exogenous P4 or steroid-rich preovulatory follicular fluid into the oviductal wall in sows increased considerably the number of spermatozoa around the oocyte and the polyspermy compared to control animals (Hunter 1972, Hunter *et al.* 1999), leading to the hypothesis that P4 might trigger sperm release from the porcine oviductal reservoir (Hunter 2008). However, the very low concentration of P4 (around 0.1 ng/mL) in the porcine oviductal fluid prior to and after ovulation (Brussow *et al.* 2008) compared to the concentrations tested in the previous studies (100 ng/mL *in vitro* and 1 mg/animal *in vivo*) do not allow to conclude on such a role. In the bovine, data on the regulation of the sperm reservoir by steroid hormones are scarce and inconsistent. *In vitro*, the number of spermatozoa that bound to bovine oviductal explants was higher before than after ovulation (Sostaric *et al.* 2008) or was not affected by the stage of the cycle (Lefebvre *et al.* 1995). We reported earlier a consistent rise in P4 concentrations (from 6 to 57 ng/mL) in parallel with a decrease in E2 concentrations (from 290 to 118 pg/mL) in the bovine oviductal fluid ipsilateral to the side of ovulation from the pre- to the postovulatory stage of the estrous cycle (Lamy *et al.* 2016). We hypothesized that P4 and E2 may regulate the attachment to and release from the sperm reservoir and select by this way a subpopulation of spermatozoa with high fertilizing competence.

Thus, the objectives of this study were to evaluate the effects of P4 and E2 at various concentrations on (1) the ability of BOEC to bind spermatozoa; (2) the release of bound spermatozoa from BOEC and (3) to investigate the fertilizing ability of spermatozoa bound to and subsequently released from BOEC.

Materials and methods

Unless specified, all chemicals were purchased from Sigma-Aldrich.

Bovine oviductal epithelial cell culture

The process of primary cell culture used in this study was already described and validated, indicating a high rate of epithelial cells (ciliated and secretory) expressing cytokeratin and showing an epithelial type ultrastructure under electron microscope (Van Lagendonck *et al.* 1995). Briefly, bovine oviducts were collected from a local slaughterhouse and transported at 37°C to the laboratory within 2-h post-mortem and processed immediately as follows. According to the morphology of ovaries and corpus luteum (Ireland *et al.* 1980), only oviducts at pre- or postovulatory stage were used. Both oviducts from a cow were cleaned from surrounding tissues and mucosae were mechanically expelled from the whole oviducts by gentle scraping with a sterile glass slide. Bovine OEC (BOEC) were then washed three times 10 min by sedimentation with HEPES-buffered tissue culture medium-199 (TCM199). The resulting cellular pellet was diluted in 10 mL of TCM199 supplemented with 10% heat-treated fetal calf serum (FCS) and 80 µg/mL gentamycin before seeding in a Lab-Tek II Chamber Slide system (Nunc, Roskilde, Denmark) and placed in a humidified atmosphere with 5% CO₂ at 38.8°C. The medium was renewed after 48 h, and then half-renewed every 48 h until cell confluence (7–8 days).

Sperm preparation and co-incubation with BOEC

A pool of frozen semen from three bulls (*Bos taurus*, 0.25-mL straws, approximately 20 × 10⁶ spermatozoa/straw) was used in all experiments. Straws were thawed in a water bath at 37°C for one minute and then washed on a discontinuous Percoll (GE Healthcare Life Sciences) density gradient (45/90%). The sperm pellet was washed and stained at the same time in 5 mL of STL-medium (Tyrode medium supplemented with 25 mM bicarbonate, 10 mM lactate, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2.4 mg/mL HEPES) containing 10 µg/mL of the blue DNA dye Hoechst 33342 at 37°C and then centrifuged at 100 g for 10 min. The final sperm pellet was resuspended in IVF-medium (Tyrode medium supplemented with 25 mM bicarbonate, 10 mM lactate, 1 mM pyruvate, 6 mg/mL fatty acid free bovine serum albumin, 100 IU/mL penicillin and 100 µg/mL streptomycin) and the concentration of spermatozoa was determined with a Thoma cell. Before being inseminated with spermatozoa, BOEC at confluence were washed once with IVF-medium and then incubated for 5 minutes with 5 µM of the fluorescent red DNA dye DRAQ5 (Deep Red Anthraquinone 5; ThermoScientific), then washed again with the same medium. All experiments of sperm-BOEC co-incubation were performed in IVF-medium at 38.8°C in a humidified atmosphere with 5% CO₂.

Quantification of bound spermatozoa

At the end of co-incubation, BOEC were vigorously washed three times with IVF-medium in order to eliminate unbound and slightly attached spermatozoa. BOEC were then fixed in 2.5% glutaraldehyde in PBS for one night at room temperature, and then rinsed in PBS and mounted under a coverslip. Slides were observed with a 20× NA 0.5 objective, using a laser-scanning confocal microscope (LSM780, Zeiss, Oberkochen,

Germany) coupled with a spectral imaging detector. The entire spectrum of fluorophores in the specimen was first gathered and then linearly unmixed. The linear unmixing was performed on Zen 2011 software (Carl Zeiss) with the 'Online fingerprinting' mode. The excitation wavelengths 405 and 633 were used for Hoechst and DRAQ5, respectively. For each experimental condition, the number of BOEC and bound spermatozoa was automatically determined by analyzing 10 randomly chosen fields of 0.18 mm² using the image processing software Fiji (Schindelin *et al.* 2012). This software surrounded all counted cells with a thin white line. The counting of all BOEC and sperm cells by the software was then checked visually. Numbers of bound spermatozoa were finally reported to 100 BOEC for each condition.

Correlative scanning-transmission electron microscopy

Interactions between spermatozoa and BOEC were observed using the correlative scanning/transmission electron microscopy (CSTEM) method previously reported for observations of cell-virus interactions (Burlaud-Gaillard *et al.* 2014). A preliminary study was conducted to determine the time course of sperm binding to BOEC: the mean number of bound sperm per 100 BOEC increased during the first 30 min of co-incubation, and then reached a plateau that was maintained for at least 120 min (data not shown). BOEC were co-incubated for 30 min with spermatozoa at a final concentration of 0.5×10^6 /mL as described previously. Samples were fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h and post-fixed with 2% osmium tetroxide in 0.15 M phosphate buffer (4% osmium solution was diluted with 0.3 M phosphate buffer) for 1 h. Samples were washed in PBS (10 min) and water (3×10 min), dehydrated in a graded series of ethanol solutions then incubated for 3 h in hexamethyldisilazane (HMDS) diluted at 50% in ethanol, then in pure HMDS for 1 h and were dried on air in a second HMDS solution. For easy orientation of samples, a coordinate grid was applied to the Lab-Tek slide surface. Dry samples were sprinkled onto carbon disks and coated with 4 nm of palladium with a GATAN PECS 682 apparatus (Pleasanton, USA) before observation under a Zeiss Ultra plus FEG-S.E.M. scanning electron microscope (Zeiss). Zones of interest were photographed at various magnifications from 30 $\times$ to 8000 $\times$. Lab-Tek slides were then separated from carbon disks with a scalpel and incubated in a mixture of absolute ethanol and EPON (3:1 in volume) for 3 h with closed caps and for 16 h with open caps and then transferred in pure EPON, which was allowed to polymerize for 24 h at 37°C and 48 h at 60°C. After polymerization, the Lab-Tek slides were separated from the EPON block by scalpel and forceps. Cells were found using coordinates on the grid and previous S.E.M. photos under a Zeiss Stemi 2000c stereo microscope and Nikon Eclipse 80i microscope connected with Nikon DS-Vi1 camera driven by NIS-ELEMENTS D 4.4 (Nikon) software. Semi-thin (0.5 μ m) and ultra-thin (70 nm) sections were made with a Leica Ultracut UCT ultramicrotome (Wetzlar, Germany) in a plane perpendicular to cell cultivation surface. Semi-thin sections were stained with toluidine blue (30 s at 60°C) and used for more precise identification of section level.

When areas of interest were at 3 microns from the edge of the block, serial ultra-thin sections were made, placed on EM one-slot grids coated with Formvar film and stained with 5% uranyl acetate for 20 min. The sections were then observed at 100 kV with a Jeol 1011 transmission electron microscope (Tokyo, Japan) connected to a Gatan digital camera driven by Digital Micrograph software (Gatan, Pleasanton, CA) for image acquisition and analysis.

Hormonal treatments

The general design of treatments applied to BOEC before and during sperm co-incubation is summarized in Fig. 1. For the following Experiments 1–4, BOEC were co-incubated with spermatozoa at a final concentration of 0.5×10^6 /mL. In all experiments, working solutions of P4 and E2 were diluted in absolute ethanol and added at a final concentration of 0.5% ethanol in culture medium. A vehicle control containing an equivalent amount of ethanol had no effect on the time course of sperm binding (data not shown).

Experiment 1 was designed to study the effects of a pretreatment of BOEC with E2 or P4 on sperm binding. BOEC monolayers were pretreated for 18 h with E2 at 1, 100 pg/mL or 100 ng/mL or P4 at 10, 100 or 1000 ng/mL. According to the concentrations of E2 and P4 previously measured in the bovine oviductal fluid in the periovulatory period (Lamy *et al.* 2016), concentrations of 100 pg/mL of E2 and of 10 and 100 ng/mL of P4 were considered to be physiological. Control samples were pretreated with an equivalent concentration of vehicle. Cells were then rinsed and co-incubated with spermatozoa for 10 or 60 min. After three washings to remove unbound spermatozoa, co-cultures were fixed for quantitative analysis of bound sperm as previously described.

Experiment 2 was designed to study the effects of E2 and P4 on sperm release from BOEC. BOEC monolayers were co-incubated with spermatozoa for 30 min. After three washings to remove unbound spermatozoa, co-cultures were treated for 60 min with E2 at 1, 100 pg/mL or 100 ng/mL, or P4 at 10, 100 or 1000 ng/mL or with heparin at 100 μ g/mL. This latter treatment was chosen as a positive control because heparin at 100 μ g/mL was previously reported to induce the release of ~100% of spermatozoa bound to BOEC monolayer within 1 h (Talevi & Gualtieri 2001). Control samples were treated with an equivalent concentration of vehicle. After three washings to remove unbound spermatozoa, co-cultures were fixed for quantitative analysis of bound spermatozoa.

Experiment 3 was a partial combination of Experiments 1 and 2 using physiological concentrations of E2 and P4. BOEC monolayers were pretreated during 18 h with E2 at 100 pg/mL, and then rinsed and co-incubated for 30 min with spermatozoa. After three washings to remove unbound spermatozoa, co-cultures were treated for 60 min with P4 at 100 ng/mL. Control samples were treated with equivalent concentration of vehicle. After three washings to remove unbound spermatozoa, co-cultures were fixed for quantitative analysis of bound spermatozoa.

Experiment 4 was designed to study the effects of E2 on the P4-induced release of spermatozoa from BOEC. BOEC monolayers were co-incubated with spermatozoa for 30 min.

Figure 1 General design of hormonal treatments applied to BOEC monolayers before or during co-incubations with spermatozoa in Experiments 1–4. BOEC, Bovine oviductal epithelial cells; E2, 17 β -estradiol; P4, progesterone; spz, spermatozoa. See Materials and methods section for details.

After three washings to remove unbound spermatozoa, co-cultures were treated for 60 min with P4 at 100 ng/mL or a combination of P4 at 100 ng/mL with E2 at 1, 100 pg/mL or 100 ng/mL. Control samples were treated with an equivalent concentration of vehicle. After three washings to remove unbound spermatozoa, co-cultures were fixed for quantitative analysis of bound spermatozoa as described above.

All experiments were repeated 3–4 times.

In vitro fertilization and embryo culture

Bovine oocytes were collected and matured *in vitro* as previously described (Cordova *et al.* 2014). Bovine ovaries were collected at a local slaughterhouse and cumulus-oocyte complexes (COCs) were collected by aspirating follicles of 2–5 mm in diameter. COCs surrounded by several layers of compact cumulus cells were selected and washed three times in HEPES-buffered TCM199. Groups of 50 COCs were then transferred into four-well dishes (Nunc) and allowed to mature for 22 h in 500 μ L of TCM199 supplemented with EGF (10 ng/mL), IGF-1 (19 ng/mL), FGF (2.2 ng/mL), hCG (5 IU/mL), PMSG (10 IU/mL), insulin (5 μ g/mL), transferrin (5 μ g/mL), selenium (5 ng/mL), L-cystein (90 μ g/mL), beta-mercaptoethanol (0.1 mM), ascorbic acid (75 μ g/mL), glycine

(720 μ g/mL), glutamine (0.1 mg/mL) and pyruvate (110 μ g/mL) at 38.8°C in a humidified atmosphere with 5% CO₂. At the end of maturation, COCs were washed three times in IVF-medium before being transferred in groups of 30–50 into four-well dishes for insemination.

The general design of sperm treatments before IVF is summarized in Fig. 2. Spermatozoa and BOEC were prepared as described above except that Hoechst and DRAQ5 staining, respectively, were omitted. BOEC monolayers were co-incubated for 30 min with spermatozoa at a final concentration of 4×10^6 spermatozoa/mL in twelve-well dishes (CytoOne, Starlab, Miltone Keynes, United Kingdom) containing 1 mL of IVF-medium per well. After co-incubation, BOEC were washed three times with IVF-medium: the cell supernatant and washing medium containing unbound spermatozoa in the presence of BOEC (BOEC-spz) were kept (38.8°C, 5% CO₂ in air). The release of bound sperm from BOEC was then induced by a treatment for 60 min with P4 at a final concentration of 100 ng/mL. BOEC were then washed three times with IVF-medium: the cell supernatant and washing medium were kept to recover the P4-released sperm subpopulation (P4-BOEC-spz). To test the effects of P4 alone, spermatozoa incubated without BOEC in IVF-medium were treated with 100 ng/mL of P4 for 60 min (P4-spz). As controls,

Figure 2 General design of sperm pretreatment before *in vitro* fertilization experiments. BOEC, Bovine oviductal epithelial cells; BOEC-spz, unbound sperm after 30 min of co-incubation with BOEC; BOEC-P4-spz, sperm bound to BOEC after 30 min of co-incubation then released by a 60-min treatment with 100 ng/mL of P4; Control spz, sperm manipulated as other treated groups; P4, progesterone; P4-spz, sperm manipulated as other treated groups and treated with 100 ng/mL of P4 during 60 min before IVF. See Materials and methods section for details.

spermatozoa were thawed and prepared just before oocyte insemination as previously described (control with vehicle) or incubated at a final concentration of 4×10^6 spermatozoa/mL in IVF-medium and manipulated by pipetting as other treated groups (control with vehicle and manipulation). The incubation of control with manipulation and P4-spz groups started at the same time as those co-incubated with BOEC. At the end of treatments, BOEC-spz, P4-BOEC-spz, P4-spz and control with manipulation were centrifuged for 10 min at 100g. Sperm pellets were pooled per condition and sperm concentrations determined with a Thoma cell. For IVF, sperm cells at a final concentration of 1×10^6 /mL were co-incubated with *in vitro*-matured oocytes at 38.8°C in 500 µL of IVF-medium containing 10 µg/mL heparin in a humidified atmosphere with 5% CO₂.

Twenty-two hours post insemination (pi), presumptive zygotes were washed three times in synthetic oviductal fluid (Holm *et al.* 1999) to remove cumulus cells and attached spermatozoa. Zygotes were then cultured in 25 µL drops of SOF supplemented with 5% heat-treated FCS and overlaid with 700 µL of mineral oil. Zygotes were incubated for 8 days at 38.8°C in a humidified atmosphere containing 5% O₂, 5% CO₂ and 90% N₂. Cleavage rates were determined on Day 2 pi. Blastocyst rates were determined on Days 7 and 8 pi as percentages of total number of COCs and total number of cleaved embryos.

Statistical analysis

Data were analyzed using the R software (version 3.2.3, tests of Foundation for Statistical Computing, Vienna, Austria). Data from Experiments 1–4 did not pass the tests for normality and homogeneity of variances (Shapiro–Wilk and Fligner–Killeen, respectively): the non-parametric Kruskal–Wallis test was used followed by Dunn’s test for multiple comparisons. In IVF experiments, the effects of treatment on cleavage and blastocyst rates were compared using chi-square analysis. The two control groups (control with and without manipulation) were not different and pooled for further analysis. Differences were considered statistically significant when $P < 0.05$.

Results

Observation of sperm–BOEC interactions

CSTEM analysis showed that sperm heads with intact acrosome interacted with both cilia and microvilli at the apical surface of BOEC (Fig. 3). Close contacts were seen between epithelial cells and the whole bottom surface of sperm heads (Fig. 3B) or only the sperm rostral acrosomal region (Fig. 3B and D).

Figure 3 Observation of sperm–BOEC interactions by correlative scanning/transmission electron microscopy. (A) Scanning electron micrograph of spermatozoa bound on BOEC microvilli; insert: sperm cells at small magnification; (B) TEM image of the same cells showing interactions between the whole bottom face (on the left) or the rostral acrosomal region (on the right) of the sperm head with microvilli; (C) S.E.M. image at high magnification of a sperm head bound to microvilli; (D) TEM image of the same sperm head, showing its bottom part interacting with BOEC microvilli. TEM sections were prepared in the plane perpendicular to cell cultivation surface: dotted lines in (A and C) show levels of section. Scale bars: 10 µm (insert in A); 5 µm (A and B); 1 µm (C and D).

Pretreatment of BOEC with E2 modified the kinetics of sperm binding

In Experiment 1, a significant decrease by 25% in the mean number of bound sperm was observed after 10 min of co-incubation with BOEC pretreated with 100 pg/mL and 100 ng/mL of E2 ($P < 0.05$), and after 60 min of co-incubation with BOEC pretreated with 100 ng/mL of E2 ($P < 0.01$; Fig. 4A). However, the pretreatment of BOEC with P4 had no effect on sperm binding after 10 and 60 min of co-incubation (Fig. 4B).

P4-induced sperm release from BOEC

In Experiment 2, heparin at 100 µg/mL induced the release of almost all spermatozoa from BOEC within 60 min (Fig. 5). Furthermore, 60-min treatments with P4 at the three concentrations tested induced a significant decrease by 32–42% in the number of bound spermatozoa (Fig. 5B). This sperm-releasing effect of P4 was more significant at the dose of 1000 ng/mL ($P < 0.01$) than at 10 and 100 ng/mL ($P < 0.05$). Conversely, E2 at the three concentrations tested did not have any effect on sperm release (Fig. 5A).

Experiment 3 showed that the sperm-releasing effect of P4 at the physiological concentration of 100 ng/mL was still evidenced after a pretreatment of BOEC with a physiological concentration (100 pg/mL) of E2 (Fig. 6).

E2 inhibited the releasing effect of P4 on bound sperm

In Experiment 4, P4 at 100 ng/mL induced a significant decrease by 47% in the number of bound sperm compared with the control group, as previously shown

Figure 4 Effects of a pretreatment of BOEC with 17β-estradiol (E2) and progesterone (P4) on sperm binding. BOEC were pretreated with E2 at 1, 100 pg/mL or 100 ng/mL (A) or with 10, 100, 1000 ng/mL of P4 (B) during 18 h before co-incubation with spermatozoa for 10 or 60 min (Experiment 1). Control cells were pretreated with vehicle. Results are expressed as mean ± S.E.M. of bound spermatozoa per 100 BOEC. ** $P < 0.01$; * $P < 0.05$.

Figure 5 Effects of treatments with 17 β -estradiol (E2) or progesterone (P4) on sperm release from BOEC. After 30 min of sperm-BOEC co-incubation, co-cultures were treated with E2 at 1, 100 pg/mL or 100 ng/mL (A), P4 at 10, 100 or 1000 ng/mL (B) or heparin at 100 μ g/mL as a positive control for 60 min (Experiment 2). Controls were treated with vehicle. Results are expressed as mean $\pm$ S.E.M. of bound spermatozoa per 100 BOEC. *** P <0.001; ** P <0.01; * P <0.05.

(P <0.001; Fig. 7). When P4 at the same concentration was added with 1 pg/mL of E2, a less but still significant decrease (by 34%; P <0.01) in the number of bound sperm was observed. However, when P4 was added with 100 pg/mL or 100 ng/mL of E2, numbers of bound sperm remained comparable to that in the control group. Thus,

Figure 6 Effect of a pretreatment of BOEC with 17 β -estradiol (E2) on the releasing effect of P4 on bound sperm. BOEC were pretreated with E2 at 100 pg/mL for 18 h before being co-incubated with spermatozoa during 30 min. Co-cultures were then treated with or progesterone (P4) at 100 ng/mL for 60 min (Experiment 3). Controls were treated with vehicle. Results are expressed as mean $\pm$ S.E.M. of bound spermatozoa per 100 BOEC. *** P <0.001.

Figure 7 Effect of 17 β -estradiol (E2) on the releasing effect of progesterone (P4) on bound sperm. After 30 min of sperm-BOEC co-incubation, co-cultures were treated with P4 at 100 ng/mL or P4 at 100 ng/mL + E2 at 1, 100 pg/mL or 100 ng/mL for 60 min (Experiment 4). Controls were treated with vehicle. Results are expressed as mean $\pm$ S.E.M. of bound spermatozoa per 100 BOEC. *** P <0.001; ** P <0.01.

E2 inhibited the P4-induced release of bound sperm from BOEC in a dose-dependent manner.

Sperm bound to BOEC then released by P4 displayed higher fertilizing ability and blastocyst yield

In Experiment 5, the cleavage rate obtained at Day 2 after insemination with P4-released spermatozoa from BOEC (P4-BOEC-spz; 86.7%) was significantly higher than that obtained in the control group (74.1%; Table 1). However, the cleavage rates of spermatozoa unbound after co-incubation with BOEC (BOEC-spz) and spermatozoa treated with P4 alone (P4-spz) did not differ from that in the control group. Furthermore, at Days 7 and 8 post insemination, the rates of blastocyst obtained with P4-BOEC-spz were higher than those in the control group (43.3% vs 30.5% and 40.4% vs 27.7% at Days 7 and 8, respectively). When calculated as percentages of cleaved embryos, the blastocyst rate obtained with P4-BOEC-spz remained significantly higher than that in the control group at Day 8 post insemination (45.9 vs 33.3%; Table 1).

Table 1 Cleavage rates at Day 2 and blastocyst rates at Days 7 and 8 after insemination of bovine *in vitro*-matured oocytes with control spermatozoa, spermatozoa treated with 100 ng/mL of progesterone (P4-spz), unbound spermatozoa after co-incubation with BOEC (BOEC-spz) or spermatozoa bound to BOEC then released by a treatment with 100 ng/mL of progesterone (BOEC-P4-spz).

Treatment	n	Cleavage rate		Blastocyst yield		
		Day 2 pi		Day 7 pi		Day 8 pi
		%	%*	%*	%**	%**
Control	391	74.1 ^a	30.5 ^a	37.9 ^a	27.7 ^a	33.3 ^a
P4-spz	130	77.7	41.5	53.5 ^b	30.8	39.6
BOEC-spz	118	83.1	41.5	50.0	34.7	41.8
BOEC-P4-spz	211	86.7 ^b	43.3 ^b	49.2	40.4 ^b	45.9 ^b

Different superscripts within columns indicate statistical differences (P <0.05, chi-squared test).

n, total number of cumulus-oocyte complexes (COCs); %, of total COCs; **%, of cleaved embryos.

Discussion

In the cow, after insemination, a sperm reservoir is formed in the oviducts, allowing the storage and then the progressive release of selected spermatozoa toward the ovulated oocyte. However, the endocrine regulation of these events has been poorly investigated. The main findings of this *in vitro* study are that: (1) a pretreatment of BOEC with E2 at physiological concentrations slightly modified the kinetics of sperm binding; (2) P4 triggered the release of bound spermatozoa from BOEC; (3) E2 inhibited the releasing effect of P4 on bound sperm in a dose-dependent manner; (4) the sequential binding to the BOEC followed by P4-induced release selected a sperm subpopulation with higher fertilizing and developmental ability than controls. These results support the hypothesis that dynamic changes in steroid hormone concentrations around the time of ovulation regulate the formation of the sperm reservoir and the subsequent delivery of capacitated spermatozoa to the site of fertilization.

The *in vitro* model of confluent BOEC monolayers used in the present study was previously shown to generate a typical display of epithelial cell morphology and cytokeratin expression (Van Lagendonck *et al.* 1995) and to be able to sustain early bovine embryo development and quality (Cordova *et al.* 2014, Schmaltz-Panneau *et al.* 2015). The same model used in the present study allowed a reliable and reproducible visualization and counting of bound spermatozoa, as previously described in numerous studies on sperm–oviduct interactions (Pollard *et al.* 1991, Gualtieri & Talevi 2000, 2003, Talevi & Gualtieri 2001, Osycka-Salut *et al.* 2017). In order to further characterize our *in vitro* model, observations of sperm–oviduct interactions after 30 min of co-incubation were carried out by CSTEM. The advantage of the recent correlative S.E.M./TEM method, applied for the first time on sperm–BOEC co-culture, is to combine in the same interacting cells the visualization of cell surface and intracellular ultrastructure (Burlaud-Gaillard *et al.* 2014). Transmission electron microscopy images showed that cell microvilli and cilia interacted either with the whole bottom surface or only with the acrosomal region of sperm cells despite very similar scanning electron microscopy pictures obtained at the cell surface, showing acrosome-intact sperm heads bound to BOEC. These pictures were in accord with earlier studies in the same species (Pollard *et al.* 1991, Suzuki & Foote 1995, Gualtieri & Talevi 2000). *In vivo*, the sperm reservoir is mainly located in the distal part of the oviduct, namely the isthmus (Hunter & Wilmut 1984). However, *ex vivo*, bull spermatozoa bind in similar patterns and in equivalent numbers to explants from the ampulla and isthmus (Lefebvre *et al.* 1995, Sostaric *et al.* 2008). Furthermore, the direct video recording of mouse sperm during their migration toward the oocyte showed that spermatozoa detach and attach again several times

to the oviduct epithelium, including the ampulla (Chang & Suarez 2012). Thus, as a first attempt to decipher the potential roles played by P4 and E2 on sperm–oviduct interactions, this simple and valuable bioassay system with BOEC from the whole oviduct was used.

It is widely accepted that the ovarian steroid hormones P4 and E2 are major regulators of the oviductal epithelium, acting through genomic and non-genomic pathways (Hunter 2012). We reported important fluctuations in the topical concentrations of P4 and E2 in the bovine oviductal fluid between the pre- and the postovulatory stages of the estrous cycle, in particular in the side of ovulation (Lamy *et al.* 2016). According to these data, concentrations of 100 pg/mL of E2 and of 10 and 100 ng/mL of P4 were considered to be representative of concentrations the apical surface of the bovine oviduct epithelium is exposed to *in vivo* around the time of ovulation. Overall, the results of Experiment 1 showed that the pretreatment of BOEC with physiological concentrations of E2 and P4 did not dramatically alter their ability to bind spermatozoa. Indeed, except with the supraphysiological concentration of 100 ng/mL of E2, the density of bound spermatozoa was the same in control and pretreated cells after 60 min of co-incubation. This is in accord with previous results showing similar numbers of spermatozoa bound to bovine oviduct explants collected at different stages of the estrous cycle in the cow (Lefebvre *et al.* 1995). Accordingly, human oviduct explants incubated overnight with physiological concentrations of E2 (Baillie *et al.* 1997) and porcine oviduct epithelial vesicles pretreated for 48 h with 100 ng/mL of E2 (Bureau *et al.* 2002) displayed similar sperm-binding capacities compared with non-treated samples after 30–60 min of co-incubation. In the present study, a pretreatment of BOEC with E2 slightly delayed the kinetics of sperm binding as less spermatozoa bound to treated cells (with 100 pg/mL and 100 ng/mL of E2) than to control cells after 10 min of co-incubation. To our knowledge, the evaluation of sperm-binding capacity within such a short interval of time after sperm co-incubation has not been reported previously. The E2 pretreatment might have modified the cell-surface proteins involved in sperm binding and modulated in this way the kinetics of sperm binding to BOEC. Nevertheless, the effect of pretreatment with 100 pg/mL of E2 on BOEC binding capacity was slight (25% decrease in numbers of bound sperm) and short lasting.

Near the time of ovulation, the sperm detachment from the oviduct epithelium and progressive migration toward the oocyte represent crucial steps for a successful fertilization. The sperm-releasing process occurs *in vivo* in parallel with an increase in topical P4 concentrations (Lamy *et al.* 2016). In the present study, P4 from 10 to 1000 ng/mL in concentration triggered sperm release from BOEC within 60 min. However, the effect of 100 µg/mL of heparin on sperm release was always stronger than that of P4: >90% vs 32–47%

of spermatozoa detached from BOEC after exposure to heparin and P4, respectively. A long (at least 4h) exposure to heparin at the optimal concentration of 5–10 µg/mL has been known for a long time to induce sperm capacitation in the bovine (Parrish *et al.* 1988). At the higher concentration of 100 µg/mL, heparin was reported to be a rapid inducer of sperm release from OEC in the bovine (Talevi & Gualtieri 2001, 2010). However, the release of sperm cells from the reservoir is a gradual process taking place over several hours (Chang & Suarez 2012): the detachment of all spermatozoa within one hour as evidenced with 100 µg/mL heparin is unlikely to happen *in vivo*. The sperm-releasing effects of P4 in the present study are in accord with previous *in vivo* results in the porcine in which an injection of P4 beneath the serosa or within the lumen of the tubal isthmus led to an increase in the number of spermatozoa around oocytes and polyspermy (Hunter 1972, 2008, Hunter *et al.* 1999). Taking into account the inhibitory effects of P4 on oviduct smooth muscle contractions and mucosa edema, this higher number of spermatozoa around oocytes was interpreted as a consequence of reduced physical barriers to sperm progression toward the site of fertilization (Suarez 2016). However, our results support a direct action of P4 on spermatozoa or simultaneously on both sperm and oviduct epithelial cell surface to trigger sperm release. It is likely that hyperactivation plays an important role in the release of spermatozoa from the oviduct epithelium in mammals (Demott & Suarez 1992, Chang & Suarez 2012, Ardon *et al.* 2016). Spermatozoa with hyperactivated motility were observed while they detached from bovine mucosal folds of oviduct epithelium *in vitro* (Ardon *et al.* 2016) and from the epithelium in entire oviducts retrieved from mouse females just after mating (Demott & Suarez 1992, Chang & Suarez 2012). Hyperactivated spermatozoa display asymmetrical, high-amplitude flagellar movements (Yanagimachi 1970) that are not only important for sperm release but also essential for the penetration of the cumulus oophorus and the zona pellucida surrounding the oocyte (Hung & Suarez 2010). Moreover, there is evidence that P4 induces or enhances hyperactivated motility in human and hamster spermatozoa (Sueldo *et al.* 1993, Noguchi *et al.* 2008), probably mediated through an influx of calcium into sperm cells (Publicover *et al.* 2007). Thus, it is likely that the sperm detachment in the present study was mediated by a P4-triggered hyperactivated motility on bound spermatozoa. However, as evoked above for E2, a modification by P4 of the cell-surface proteins that would reduce the binding affinity of spermatozoa cannot be excluded. The expression of nuclear as well as membrane P4 receptor membrane components (PGRMC) 1 and 2 were previously shown in BOEC (Saint-Dizier *et al.* 2012). Taking into account the relatively short time (60 min) during which sperm-BOEC co-cultures were exposed to P4, these cell-surface

modifications, if they occur, would be mediated through the latter by non-genomic mechanisms.

In the present study, E2 had no effect on sperm release by itself, as shown in Experiment 2. A 18-h pretreatment of BOEC with 100 pg/mL of E2, that was applied to mimic the peri-ovulatory action of E2 on oviductal cells, did not affect the releasing action of P4 on bound sperm, as shown in Experiment 3. However, in Experiment 4, E2 acted as a suppressor of the sperm-releasing effect of P4 when added simultaneously with this hormone. This effect of E2 was dose dependent: the releasing effect of 100 ng/mL of P4 was suppressed by ≥100 pg/mL but not with 1 pg/mL of E2. Similarly in hamster spermatozoa, E2 did not affect sperm motility by itself but suppressed P4-stimulated hyperactivation in a concentration-dependent manner: this effect was mediated by the inhibition of tyrosine phosphorylation through non-genomic pathways (Fujinoki 2010). Taken together, it could be hypothesized that E2 prevented the P4-induced sperm release from BOEC above 100 pg/mL by a dose-dependent inhibition of sperm hyperactivation. Further work examining the sperm motility and intracellular physiology at the time of addition of P4 or P4 + E2 would be necessary to support this hypothesis. Furthermore, diethylstilbestrol, an estrogen-like product, has been previously shown to disrupt the effects of P4 and E2 on sperm hyperactivation (Fujinoki 2014): it would be of interest to examine the effect of estrogen-like products on the regulation of sperm release by P4 and E2 in the bovine.

It is noteworthy that the concentration at which E2 exerted its inhibitory effect was very close to the concentration (118 pg/mL) that was previously reported in the bovine tubal fluid after ovulation (Lamy *et al.* 2016), at which time sperm release is supposed to happen. Nevertheless, this value is an average concentration measured in pools of fluids collected between Days 1–5 of the estrous cycle (Lamy *et al.* 2016) and may not reflect the fine-tuned hourly changes in the P4:E2 balance that probably occur within the oviduct around the time of ovulation. The postovulatory decline in topical E2 might lengthen the release of spermatozoa from the sperm reservoir and thus prolong the fertilization time window *in vivo*.

Finally, in order to better characterize the P4-released spermatozoa from BOEC, *in vitro* fertilization experiments were undertaken. The results showed that the binding to the BOEC followed by the P4-induced release from BOEC enhanced the ability of spermatozoa to fertilize oocytes compared with spermatozoa in the control group. This is in accord with previous results showing that the binding to OEC achieves the selection of a sperm subpopulation with intact acrosome and normal morphology (Thomas *et al.* 1994, Lefebvre & Suarez 1996, Ellington *et al.* 1999a, Gualtieri & Talevi 2000). These results also agree with a previous study in which bull spermatozoa retrieved from

BOEC co-culture with an heparin treatment displayed enhanced ability to adhere to the zona pellucida and to fertilize oocytes compared with unbound sperm (Gualtieri & Talevi 2003). In addition to its role on sperm hyperactivation, stimulating roles of P4 were reported on mammalian sperm capacitation, acrosome reaction and chemical guidance (Baldi *et al.* 2009, Guidobaldi *et al.* 2012, Fujinoki *et al.* 2016). The exposition to P4 alone in the absence of BOEC did not have any significant effect on sperm fertilizing ability compared with the control group. However, no difference in the cleavage rates was either seen between spermatozoa exposed to P4 alone and those previously bound to BOEC and released by P4. It could be hypothesized that spermatozoa with high fertilizing competence were selected by a double check point: a first selection was achieved by the adhesion to BOEC, then the ability to respond to the P4 signal by detaching from BOEC, displayed by 32–47% of spermatozoa in Experiments 2–4, represented a second selection step. The binding to BOEC followed by the P4-induced release improved not only the ability of sperm to bind and penetrate oocytes, reflected by the cleavage rate, but also improved the embryo development up to the blastocyst stage at Day 8 compared with the control group. It is well established that pretreatment or preparation allowing the selection of a sperm subpopulation with high motility, membrane and/or DNA integrity support embryo development and blastocyst yield after IVF in the bovine (Jaakma *et al.* 1997, Gualtieri *et al.* 2014, Pang *et al.* 2016). Our results support the hypothesis that the ability to bind to BOEC and to be released by P4 is a highly selective process for bull spermatozoa. A previous study reported a positive association between the ability of sperm to bind OEC *in vitro* and the *in vivo* fertility in bulls (De pauw *et al.* 2002). Our *in vitro* system may be useful to determine whether the sequential binding to the BOEC followed by the P4-induced release from the BOEC could be of help in better predicting the *in vivo* fertility of a given bull.

Conclusion

In conclusion, we characterized some crucial roles played by the steroid hormones P4 and E2 in the regulation of sperm–oviduct interactions in a bovine *in vitro* model. Progesterone triggers sperm release from the oviduct epithelium and selects in this way a subpopulation of spermatozoa with high fertilizing competence, while E2 inhibits P4-induced sperm release. Further work is now needed to clarify how the dynamic topical E2:P4 balance in the periovulatory period orchestrates sperm attachment and release *in vivo* and to identify the specific mechanisms of action of these hormones on sperm physiology and/or on modulation of molecules on the surface of tubal cells.

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.

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