REPRODUCTION
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Bovine oviduct epithelial cells downregulate phagocytosis of sperm by neutrophils: prostaglandin E_2 as a major physiological regulator

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Abstract

This study aimed to investigate the presence of polymorphonuclear neutrophils (PMNs) in bovine oviduct fluid under physiological conditions and to determine the possible role of bovine oviduct epithelial cells (BOECs) in the regulation of the phagocytic activity of PMNs for sperm. During the pre-ovulatory stage, PMNs were identified in the bovine oviduct fluid in relatively constant numbers. In our experiments, PMNs were incubated for 4 h with the supernatant of cultured BOECs stimulated for 24 h by LH (10 ng/ml). Phagocytosis was then assayed by co-incubation of these PMNs with sperm treated to induce capacitation. The BOEC supernatant significantly suppressed sperm phagocytosis by PMNs, and the LH-stimulated BOEC supernatant further suppressed phagocytosis. Importantly, in the BOEC culture, LH stimulated the secretion of prostaglandin E_2 (PGE_2), which dose-dependently (10^{-6}, 10^{-7}, and 10^{-8} M) suppressed sperm phagocytosis by PMNs. Furthermore, a $PGEP_2$ receptor antagonist significantly abrogated the inhibition of phagocytosis by the LH-stimulated BOEC supernatant. Additionally, using scanning electron microscopy, incubation of PMNs with either PGE_2 or LH-stimulated BOEC supernatant before phagocytosis was found to prevent the formation of DNA-based neutrophil extracellular traps for sperm entanglement. The results indicate that sperm are exposed to PMNs in the oviduct and PGE_2 released into the oviduct fluid after LH stimulation may play a major role in the suppression of the phagocytic activity of PMNs for sperm via interaction with EP_2 receptors. Thus, the bovine oviduct provides a PGE_2-rich microenvironment to protect sperm from phagocytosis by PMNs, thereby supporting sperm survival in the oviduct.

Free Japanese abstract

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Introduction

The oviduct plays a pivotal role in mammalian reproduction, providing an optimal environment for oocyte maturation, sperm capacitation, fertilization, and gamete and embryo transport (Ellington 1991, Hunter 2012). Anatomically, the oviduct is a thin convoluted tube that opens into the uterine horn on one end and into the peritoneal cavity on the other end. Therefore, the oviduct milieu represents a unique immunological site that supports a delicate balance between protecting the oviduct from infection by potentially pathogenic ascending microorganisms and maintaining a permissive environment for the survival of allogeneic sperm and semi-allogeneic embryos. However, little is known about how the oviduct immune system interacts with the allogeneic sperm and semi-allogeneic embryos.

Oviduct epithelial cells secrete a variety of molecules, including oviductal glycoproteins (Abe *et al.* 1995), bicarbonate ions, and oviductin (Boatman 1997), that provide an optimal environment for sperm survival in the oviduct (Parrish *et al.* 1989, Ellington 1991). Luteinizing

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hormone (LH) is able to directly regulate the proliferative and secretory functions of the bovine oviduct through binding to its receptors on bovine oviduct epithelial cells (BOECs) (Sun *et al.* 1997, Mishra *et al.* 2003). In addition, LH stimulates the production of vasoactive peptides and angiogenic factor and vascular endothelial growth factor, which in turn stimulate the secretion of prostaglandin (PG) and tubal contraction in the bovine oviduct (Wijayagunawardane *et al.* 2001*a*, 2001*b*, 2005). These studies reveal that LH basically stimulates various secretions from BOECs, thereby inducing PG secretion.

After insemination in cows, either naturally or artificially, large numbers of sperm (several billions or $5–40\times10^6$) are deposited in the vagina or uterine body respectively (Lopez-Gatius 2000, Vishwanath 2003, Suarez 2007), but only a limited number (hundreds to thousands) reach the oviduct (Mitchell *et al.* 1985, Sostaric *et al.* 2008). Most of them are lost in the vagina, cervix, or uterus by backflow, degradation, and phagocytosis by polymorphonuclear neutrophils (PMNs; Strzemienski 1989). Insemination triggers a massive recruitment of PMNs into the uterine lumen (Mattner 1968, Strzemienski 1989, Alghamdi *et al.* 2009). The mechanism by which neutrophils phagocytize sperm is similar to that for bacteria (Alghamdi & Foster 2005). Thus, neutrophils either directly phagocytize sperm through cell–cell attachment or entrap them with neutrophil extracellular traps (NETs), structures consisting of neutrophil nuclear DNA and associated proteins, which ensnare sperm and hinder their motility (Alghamdi & Foster 2005, Alghamdi *et al.* 2009). On the other hand, it has been shown that equine seminal plasma (SP) prevents the formation of NETs (Alghamdi & Foster 2005, Alghamdi *et al.* 2009). Moreover, it has been found by Doty *et al.* (2011) that CRISP3 protein in equine SP is the factor that suppresses PMN and sperm binding and regulates sperm degradation.

Once sperm escape from phagocytosis by uterine PMNs and reach the oviduct, sperm reservoirs are formed where sperm undergo capacitation (Suarez 2008). Thus, the oviduct provides a microenvironment for sperm capacitation (Hunter & Nichol 1986, Rodriguez-Martinez 2007). After capacitation, sperm are sequentially released from the reservoirs (Hunter & Léglise 1971, Suarez 2008) and are rapidly transported to the fertilization site where the oviduct microenvironment supports the viability of sperm population for more than 24 h until ovulation. Sperm viability until ovulation is manifested by prolonged motility and fertility (Talevi & Gualtieri 2004, Chang & Suarez 2012). It has been shown that in humans (Haney *et al.* 1983), cats (Murakami *et al.* 1985), and mice (Chakraborty & Nelson 1975) the superfluous sperm that remain in the oviduct after ovulation are eliminated through phagocytosis by epithelial and immune cells. However, in cows, the presence of such immune cells, their interaction with stored sperm, and the possible regulators of the local immune microenvironment in the oviduct have not been investigated yet. We have recently provided evidence that BOECs efficiently control the balance between Th1 and Th2 cytokines (Kowsar *et al.* 2013). As such, in the present study, we investigated the presence of PMNs in the bovine oviduct fluid and the possible role of oviduct epithelial cells in the regulation of phagocytic activity of PMNs for sperm.

Materials and methods

Oviduct collection and preparation

Oviducts along with their ipsilateral ovaries were collected from a local slaughterhouse, and they were perfectly closed from both ends to prevent the leakage or contamination of oviductal contents. The stage of estrous cycle was determined macroscopically by ovarian morphology by observing the color, size, and weight of the corpus luteum as described previously (Wijayagunawardane *et al.* 1998). Furthermore, oviducts as well as attached uteri were macroscopically examined to be healthy and free from any infections. After that, oviducts were immersed in PBS without calcium or magnesium ($PBS^{-/-}$) supplemented with 0.3% gentamicin (Sigma–Aldrich) and amphotericin B (Sigma-Aldrich) and transported to the laboratory. In the laboratory, oviducts were cut, separated from the connective tissue, and externally rinsed three times with $PBS^{-/-}$.

Identification of PMNs in the oviduct fluid

Within 15 min of killing in the slaughterhouse, 14 oviducts at pre-ovulatory stage were collected. Oviducts were perfectly closed from the uterine end and then separated by making an incision through the uterine horn 10 cm away from the uterotubal junction. Oviducts were separated from the surrounding connective tissue. A blunt needle (20 gauge) was inserted from the uterine end of the oviducts and gently flushed with $PBS^{-/-}$ (2 ml/oviduct), and the resultant fluid was pooled in a sterile tube. In the laboratory, leukocytes were isolated from the oviduct flushes according to the protocol of Cotter & Muruve (2006) with minor modifications. Basically, the collected fluid was passed through a 40 µm pore cell strainer (BD Biosciences, Durham, NC, USA) and centrifuged at 300 ***g*** for 6 min at 20 °C. To purify leukocytes, the cell pellet was then suspended in 10 ml of 35% Percoll (Sigma–Aldrich) and centrifuged at 360 ***g*** for 10 min at 20 °C. After supernatant removal, the leukocyte pellet was washed with 5 ml $PBS^{-/-}$ and suspended in 1 ml $PBS^{-/-}$. Giemsa-stained PMNs were detected using a light microscope (two to five nuclear and finely granular lobes). For a total cell count, a sample of the leukocyte suspension was diluted (1:10) with 0.1% acetic acid (Sigma–Aldrich) and mounted on a hemocytometer. To determine PMN proportions in leukocyte populations, a 20 µl sample of the leukocyte suspension was diluted in MACS separation buffer (MACS Miltenyi Biotec, Tokyo, Japan) and analyzed by flow cytometric evaluation (Beckman Coulter, Inc., Miami, FL, USA).

Culture of BOECs and hormonal stimulation

The isolation and culture of BOECs were based on the method described previously (Wijayagunawardane *et al.* 1999, 2005) with minor modifications. Briefly, BOECs were mechanically dislodged, purified, and cultured in DMEM/F12 (Gibco; supplemented with 2.2% $NaHCO_3$, 0.1% gentamicin, 1% amphotericin, and 10% FCS; BioWhittaker, Walkersville, MD, USA) in six-well culture dishes (Nalge Nunc International, Roskilde, Denmark) until monolayer formation. The cells were incubated at 38.5 °C in 5% CO_2 and 95% air, and these incubation conditions were kept constant for all the cells (BOECs, sperm, and PMNs) throughout all the experiments. After monolayer formation, the cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH, USA), re-plated in six-well culture dishes at a density of 3×10^4 cells/ml, and cultured until subconfluence. The growing BOEC monolayer was then cultured in a medium supplemented with 0.1% FCS and incubated for 24 h with LH (10 ng/ml, USDA-bLH-B6, NHPP, Animal Hormone Program, Bethesda, MD, USA). Finally, the culture medium was collected and stored at −80 °C until use. The cells were collected, and cell viability was estimated using Trypan Blue staining and was confirmed to be more than 95% at each time point of plating as well as at the end of the experiment. The purity of BOECs was confirmed by anti-cytokeratin 1 immunostaining and by characteristic epithelial morphology.

Preparation of PMNs

Isolation of PMNs

PMNs were isolated as described previously (Jiemtaweeboon *et al.* 2011) with minor modifications. Blood collection experiments were conducted at the Field Center of Animal Science and Agriculture of Obihiro University, and all the experimental procedures were carried out in compliance with the Guidelines for the Care and Use of Agricultural Animals at Obihiro University. Heparinized blood from a multiparous Holstein cow in luteal stage was collected and mixed with an equal volume of $PBS^{-/-}$, slowly layered over Ficoll-Paque solution (Lymphoprep, Axis Shield, Oslo, Norway), and centrifuged at 1000 ***g*** for 30 min at 10 °C. PMN layer was mixed with ammonium chloride lysis buffer (NH_4Cl, 155 mM; $KHCO_3$, 3.4 mM; and EDTA, 96.7 μM) for 10 s and then centrifuged at 500 ***g*** for 10 min at 10 °C to purify PMNs from red blood cells. After centrifugation, the cell pellet was washed two times with $PBS^{-/-}$. The purity of PMNs as evaluated by flow cytometry was >98%, and the viability was around 99% as assessed by Trypan Blue staining.

Incubation of PMNs

Before the phagocytosis assay, PMNs were suspended at a density of 1×10^7 cells/ml with BOEC supernatant in a culture tube and incubated for 4 h with gentle shaking. The supernatant of BOECs either with or without LH stimulation was obtained by centrifugation of BOEC culture medium at 1000 ***g*** for 10 min to remove any cells or debris. The most upper 70–80% of the supernatant was used for PMN culture. After PMN incubation, PMNs were washed two times with $PBS^{-/-}$ and used for the phagocytosis assay.

Preparation of sperm

In parallel with PMN preparation, sperm preparation was carried out. Frozen semen straws were obtained from three highly fertile Holstein bulls of Genetics Hokkaido Association (Hokkaido, Japan). All semen straws were obtained from a single ejaculate from each bull separately. *In vitro* capacitation of bull sperm was induced using modified Tyrode's albumin, lactate, and pyruvate medium (Sp-TALP), according to the method described previously (Parrish *et al.* 1988, 1989) with minor modifications. Briefly, two semen straws from each bull were thawed in a cytothaw for 60 s and mixed in 5 ml Sp-TALP. Then, 1-h swim-up was carried out to obtain highly active and motile sperm. Sperm concentration was adjusted to 50×10^6 sperm/ml in Sp-TALP, and sperm were capacitated by 4 h of incubation in Sp-TALP medium supplemented with 10 μg/ml heparin. Capacitation was confirmed by the induction of acrosome reactions using 100 μg/ml lysophosphatidylcholine for 15 min. Acrosome reactions were detected by performing a dual staining procedure with Trypan Blue supravital stain and Giemsa stain as described by Kovacs & Foote (1992). After capacitation, sperm were washed and suspended in Tyrode's medium containing lactate, pyruvate, and HEPES (TL-HEPES; Bavister *et al.* 1983, Guthrie *et al.* 2002) and were then used for the phagocytosis assay. The term 'treated sperm' is used throughout the article to refer to sperm treated to induce capacitation.

Phagocytosis assay

Phagocytosis assay of sperm phagocytosis by PMNs was performed according to the method of Matthijs *et al.* (2000) with minor modifications. Briefly, the 4-h incubated PMNs were suspended in TL-HEPES. PMN suspension was mixed with sperm suspension and serum in a 96-well untreated polystyrene microtest plate (Thermo Scientific, Roskilde, Denmark) and incubated for 60 min with gentle swirling on a test-plate shaker. The final concentrations of PMNs, sperm, and fresh serum were 8×10^6, 4×10^6 cells/ml, and 12% (v/v) respectively, and the total volume was 100 μl. After incubation, an equal volume of heparin (40 mg/ml in TL-HEPES) was added to facilitate the dissociation of agglutinated PMNs. Subsamples of 75 μl were fixed by adding 25 μl of 2% (v/v) glutaraldehyde. The fixed samples were mounted on the glass slides and examined under a phase-contrast microscope at ×400 magnification connected to a digital camera and a computer system (Leica Application Suite, Germany). Minimally, 400 PMNs were counted in different areas of the specimen. The percentage of PMNs with phagocytized sperm was recorded as phagocytosis rate. Quantification of the number of PMNs with phagocytized sperm was carried out independently by two observers.

PGE_2 concentration determination

PGE_2 concentrations were measured directly in the oviduct fluid and BEOC supernatant using second antibody enzyme immunoassay as described previously (Wijayagunawardane

et al. 1998). In this experiment, 14 oviducts (pre-ovulatory, $n=4$; post-ovulatory, $n=5$; and mid-luteal, $n=5$) and 12 BOEC supernatants (with LH stimulation, $n=6$ and without LH stimulation, $n=6$) were used. The values of coefficients of variance within assay and between assay were 7.3 and 11.4% respectively. The ED_{50} values were 260 pg/ml and the ranges of the standard curves for these assays were 20–20 000 pg/ml.

Scanning electron microscopy

Neutrophils (1×10^7 cells/ml) were incubated in culture medium without any stimulus, with PGE_2 (10^{-7} M), or with LH-stimulated BOEC supernatant for 4 h, and phagocytosis was assayed. For scanning electron microscopy (SEM), each sample after phagocytosis was put on a cover glass coated with 0.1% neoprene in toluene, dried at room temperature, and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After fixation, the samples were washed in PB, post-fixed in 1% osmium tetroxide in PB, and dehydrated in a graded series of ethanol. The specimens were then freeze-dried with t-butyl alcohol using a freeze dryer (ES-2030, Hitachi). Each dried sample was mounted on a specimen stub with the cover glass and sputter coated with platinum (ion sputter coater E-1045, Hitachi). The specimens were observed using a scanning electron microscope (S3400N, Hitachi) at an accelerating voltage of 5 kV.

Experimental design

Effect of BOEC supernatant on the phagocytic activity of PMNs for sperm

PMNs were incubated in the BOEC supernatant either with or without LH stimulation for 4 h. Furthermore, PMNs were incubated in fresh medium and kept as control. As long as LH was one of the constituents of the LH-stimulated BOEC supernatant, another group was prepared by direct stimulation of PMNs with LH (10 ng/ml in fresh medium). After 4 h of incubation of PMNs, phagocytosis of treated sperm was assayed.

Dose-dependent effect of PGE_2 on the phagocytic activity of PMNs for sperm

To investigate the effects of PGE_2 on the phagocytic activity of PMNs for sperm, PMNs were exposed to different concentrations of PGE_2 (10^{-6}, 10^{-7}, and 10^{-8} M; Sigma–Aldrich) for 4 h. Then, phagocytosis of treated sperm was assayed.

Effect of EP_2 receptor antagonist on the phagocytic activity of PMNs for sperm

An EP_2 receptor antagonist (AH6809, Cayman Chemical, Ann Arbor, MI, USA) was used to block the effect of PGE_2 on the phagocytic activity of PMNs for sperm. First, PMNs were cultured with AH6809 (10^{-5} M) alone for 1 h, followed by further 3 h of incubation together with PGE_2 (10^{-7} M). Additionally, in the same way, AH6809 (10^{-5} M) was cultured with the BOEC supernatant. Then, phagocytosis of treated sperm was assayed.

Statistical analyses

Data are presented as means ± S.E.M. of three to eight experiments. Statistical analyses were carried out using StatView 5.0 (SAS Institute, Inc., Cary, NC, USA). Statistical significance between the groups was determined using *t*-test (for two groups) or one-way ANOVA followed by multiple comparison test (Fisher's test for three groups and Bonferroni's test for more than three groups), and all results were considered to be statistically significant at $P<0.05$.

Results

Identification of PMNs in the oviduct fluid

Little is known about the oviduct local immunological microenvironment including PMNs and how oviductal PMNs interact with sperm. To determine the existence of PMNs in the oviduct, we collected immune cells from the oviduct lumen by flushing it with $PBS^{-/-}$ within 15 min of killing. During the pre-ovulatory stage, PMNs were present in the bovine oviduct fluid in constant numbers (average $3–5\times10^3$ cells/oviduct flush), constituting ~17% of the total leukocyte population in the oviduct flush, whereas lymphocytes represented ~23% of the total leukocyte count (Fig. 1).

Effect of BOEC supernatant on the phagocytic activity of PMNs for sperm

Oviduct provides the optimal environment for sperm survival (Ellington 1991). Therefore, this experiment was conducted to investigate the possible role of oviduct epithelial cells in the regulation of the phagocytic activity of PMNs for sperm. No differences were observed in the phagocytic activity of PMNs for sperm throughout the cycle (data not shown). Thus, all blood samples were collected during the luteal stage for the series of experiments. Direct incubation of PMNs with LH, 10 ng/ml, for 4 h before the phagocytosis assay did not affect their phagocytic activity for sperm (Fig. 2). Meanwhile, incubation of PMNs in the BOEC supernatant resulted in a significant decrease ($P<0.05$) in their phagocytic activity for the treated sperm. Furthermore, incubation in the LH-stimulated BOEC supernatant resulted in a further decrease ($P<0.001$) in their phagocytic activity.

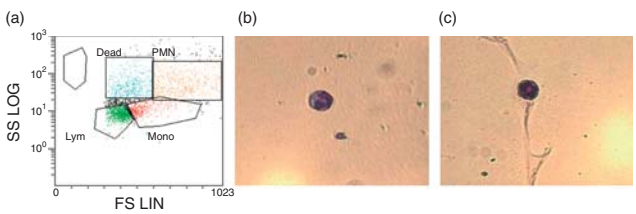

Figure 1 (a) Flow cytometric analysis of PMNs ratio in the oviduct fluid. (b and c) Light micrographs (×400) of PMNs in the oviduct fluid (two to five nuclear and finely granular lobes).

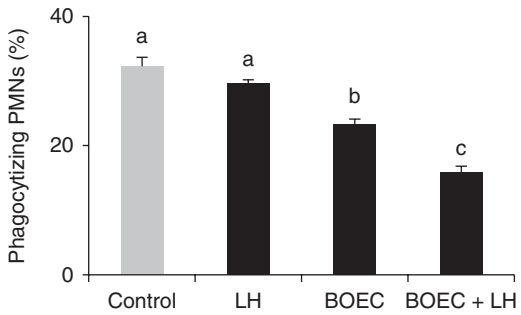

Figure 2 Percentage of *in vitro* phagocytosis of sperm treated to induce capacitation by PMNs. PMNs were incubated for 4 h in culture medium supplemented with LH (10 ng/ml), supernatant of BOEC culture, or supernatant of the LH-stimulated BOEC culture (BOEC + LH) before the phagocytosis assay. Numerical values are presented as means ± s.e.m. of eight experiments. The different letters indicate a significant difference between the marked treatments at $P<0.05$.

PGE_2 concentrations in the oviduct fluid and BOEC culture

PGE_2 is known as an immunosuppressive factor (Kalinski 2012). Thus, this experiment was conducted to determine PGE_2 concentrations in the oviduct fluid during the peri-ovulatory period and in the LH-stimulated BOEC supernatant. In the oviduct fluid (Fig. 3a), PGE_2 concentrations during the pre-ovulatory ($P<0.03$) and post-ovulatory ($P<0.06$) stages were three to four times higher than that during the mid-luteal stage. In the BOEC supernatant (Fig. 3b), the ratio of PGE_2 secretion by BOECs (16.2 ± 3.4 ng/ml, mean ± s.e.m.) was significantly ($P<0.05$) enhanced by LH stimulation.

Dose-dependent effect of PGE_2 on the phagocytic activity of PMNs for sperm

It has been shown that PGE_2 inhibits the phagocytic activity of neutrophils in guinea pigs (Smith 1977). This experiment was conducted to investigate the effects of PGE_2 as a major secretory product of BOECs on the phagocytic activity of PMNs for sperm. Incubation of PMNs with PGE_2 (10^{-6} M = 352 ng/ml, 10^{-7}, and 10^{-8} M) for 4 h before the phagocytosis assay resulted in a dose-dependent decrease in the phagocytic activity of PMNs for the treated sperm (Fig. 4). These concentrations were considered comparable to the PGE_2 concentrations in the oviduct fluid (10^{-8} M = 3.52 ng/ml) and BOEC supernatant (10^{-7} M = 35.2 ng/ml) detected in our experiment and those in the oviduct tissue extract (10^{-6} M = 352 ng/ml) previously detected by Wijayagunawardane *et al.* (1998).

Effect of EP_2 receptor antagonist on the phagocytic activity of PMNs for sperm

PGE_2 has been shown to suppress the functions of human neutrophils via EP_2 receptors (Talpain *et al.* 1995, Burelout *et al.* 2004). Thus, this experiment was conducted to investigate whether PGE_2 is one of the factors involved in the suppressive activity of the BOEC supernatant on sperm phagocytosis by PMNs. The EP_2 receptor antagonist significantly ($P<0.05$) abrogated the suppressive effect of PGE_2 on sperm phagocytosis by PMNs (Fig. 5a). Moreover, incubation of PMNs in the LH-stimulated BOEC supernatant supplemented with EP_2 receptor antagonist significantly ($P<0.05$) reversed the supernatant's inhibitory effect on sperm phagocytosis by PMNs (Fig. 5b).

Observation of NETs formation by SEM

Neutrophils either directly phagocytize sperm through cell–cell attachment or entrap them with NETs and the latter is mainly detected by SEM (Alghamdi & Foster 2005). Therefore, SEM was used to investigate the effect of the BOEC supernatant on NETs formation by PMNs for sperm entanglement. During the phagocytosis assay, the addition of sperm to PMNs induced NETs formation (Fig. 6c and d) compared with that without sperm (Fig. 6a and b). Incubation of PMNs with either PGE_2 (10^{-7} M; Fig. 6e and f) or LH-stimulated BOEC supernatant (Fig. 6g and h) before the phagocytosis assay resulted in the reduction of NETs formation by PMNs for sperm entanglement.

Discussion

The present data indicate that relatively constant numbers of PMNs exist in the oviduct fluid during the pre-ovulatory stage under physiological conditions, suggesting that the oviductal PMNs contribute to the local innate immunity mechanisms that protect the oviduct from potentially pathogenic microorganisms. These results together with the fact that the oviduct

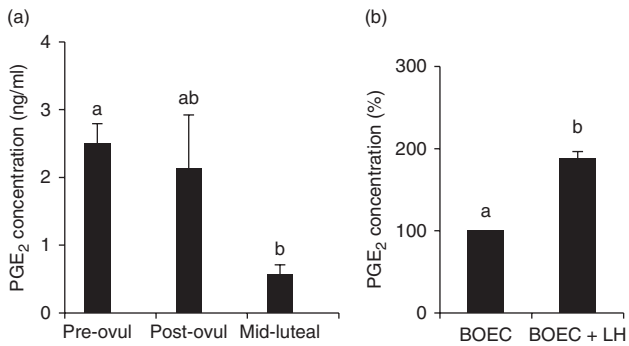

Figure 3 (a) PGE_2 concentrations (ng/ml) in the oviduct fluid during estrous cycle: pre-ovul, pre-ovulatory phase ($n=4$); post-ovul, post-ovulatory phase ($n=5$); and mid-luteal phase ($n=5$). (b) Percentage of PGE_2 concentrations in the BOEC culture stimulated with 10 ng/ml LH (BOEC + LH) or without any stimulant (BOEC) (100% = 16.2 ± 3.4 ng/ml, means ± s.e.m.). Numerical values are presented as means ± s.e.m. of four to six experiments. The different letters indicate a significant difference between the marked treatments at $P<0.05$.

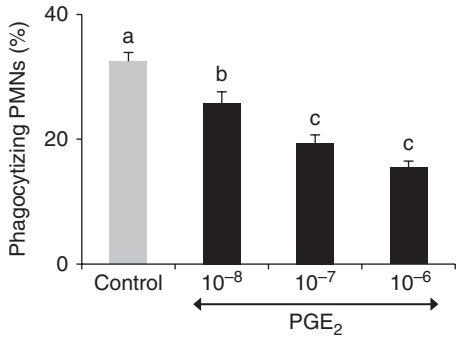

Figure 4 Dose-dependent effect PGE_2 on the percentage of *in vitro* phagocytosis of sperm treated to induce capacitation by PMNs. PMNs were incubated for 4 h in culture medium supplemented with different concentrations of PGE_2 (10^{-6} M=352 ng/ml, 10^{-7} M=35.2 ng/ml, and 10^{-8} M=3.52 ng/ml) before the phagocytosis assay. Numerical values are presented as means±s.e.m. of six experiments. The different letters indicate a significant difference between the marked treatments at $P<0.05$.

creates the optimal microenvironment for the survival of allogeneic sperm prompted us to investigate the effect of the oviduct secretions on the phagocytic activity of PMNs for sperm.

The results indicated that incubation of PMNs with the BOEC supernatant, for 4 h before the phagocytosis assay, resulted in the suppression of their phagocytic activity for the treated sperm. Moreover, incubation of PMNs with the LH-stimulated BOEC supernatant caused further reduction of the phagocytic activity of PMNs for sperm. Using SEM, we found that the LH-stimulated BOEC supernatant not only suppressed the phagocytic activity of PMNs for sperm but also reduced the formation of NETs, leading to the protection of sperm from being trapped and degraded by PMNs. The present findings suggest that factors secreted by BOECs can suppress the phagocytic activity of PMNs for the treated sperm via reduction of NETs formation, especially during the pre-ovulatory stage characterized by the increased LH levels (LH surge). Thus, BOECs work on the phagocytic activity of oviductal PMNs to create an optimal microenvironment for the survival of sperm.

In fact, we found that PGE_2 levels in the oviduct fluid during the peri-ovulatory stage were three to four times higher than that during the mid-luteal stage and LH stimulated PGE_2 secretion in the BOEC culture. PGE_2 is an immunosuppressive factor (Kalinski 2012) that has been shown to inhibit the phagocytic activity of neutrophils and macrophages in guinea pigs (Smith 1977) and mice (Aronoff *et al.* 2004) respectively. Thus, we speculated that the PGE_2 secreted by BOECs is one of the factors that contribute to the suppressive effect of the BOEC supernatant on the phagocytic activity of PMNs for sperm. In support of this idea, the results indicated that PGE_2 dose dependently suppressed sperm phagocytosis by PMNs. Additionally, SEM analysis demonstrated that PGE_2 significantly reduced the formation of NETs and suppressed the phagocytosis of sperm by PMNs, as observed in PMNs treated with the LH-stimulated BOEC supernatant. Moreover, sperm stimulate the secretion of PGE_2 by cultured BOECs (Kodithuwakku *et al.* 2007). Thus, in addition to the action of LH, sperm may have a self-protective mechanism by the suppression of the phagocytic activity of PMNs through further enhancement of PGE_2 secretion in the microenvironment surrounding them.

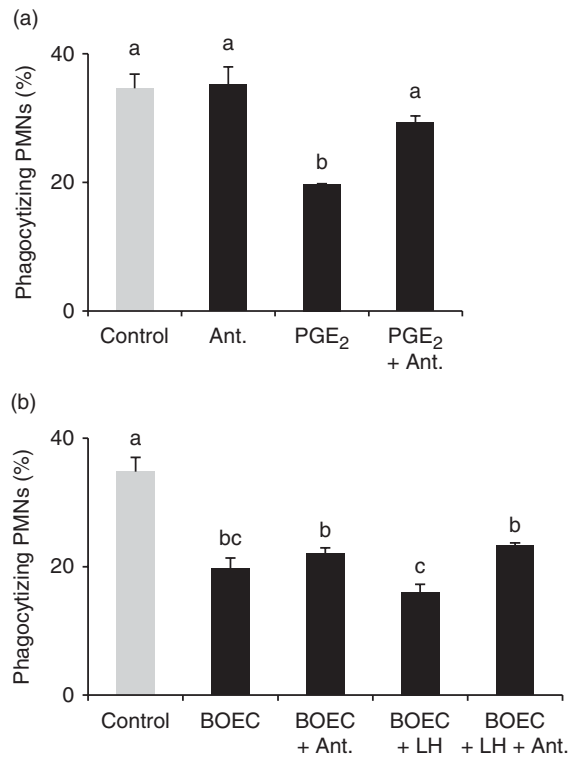

Figure 5 (a) Effect of EP_2 receptor antagonist on PGE_2-induced inhibition of phagocytosis of sperm treated to induce capacitation by PMNs *in vitro*. PMNs were incubated in culture medium supplemented with EP_2 receptor antagonist (10^{-5} M) for 4 h (Ant.), PGE_2 (10^{-7} M) for 4 h, or EP_2 receptor antagonist (10^{-5} M) for 1 h followed by 3 h of incubation together with 10^{-7} M PGE_2 (PGE_2+Ant.) before the phagocytosis assay. Numerical values are presented as means±s.e.m. of three experiments. The different letters indicate a significant difference between the marked treatments at $P<0.05$. (b) Effect of EP_2 receptor antagonist on BOEC supernatant-induced inhibition of phagocytosis of sperm treated to induce capacitation by PMNs *in vitro*. PMNs were incubated with the supernatant of BOEC culture for 4 h and EP_2 receptor antagonist (10^{-5} M) for 1 h followed by 3 h of incubation together with the supernatant of BOEC culture (BOEC+Ant.), the supernatant of LH-stimulated BOEC culture (BOEC+LH) for 4 h, or the EP_2 receptor antagonist (10^{-5} M) for 1 h followed by 3 h of incubation together with the supernatant of LH-stimulated bovine oviduct epithelial cell culture (BOEC+LH+Ant.) before the phagocytosis assay. Numerical values are presented as means±s.e.m. of three experiments. The different letters indicate a significant difference between the marked treatments at $P<0.05$.

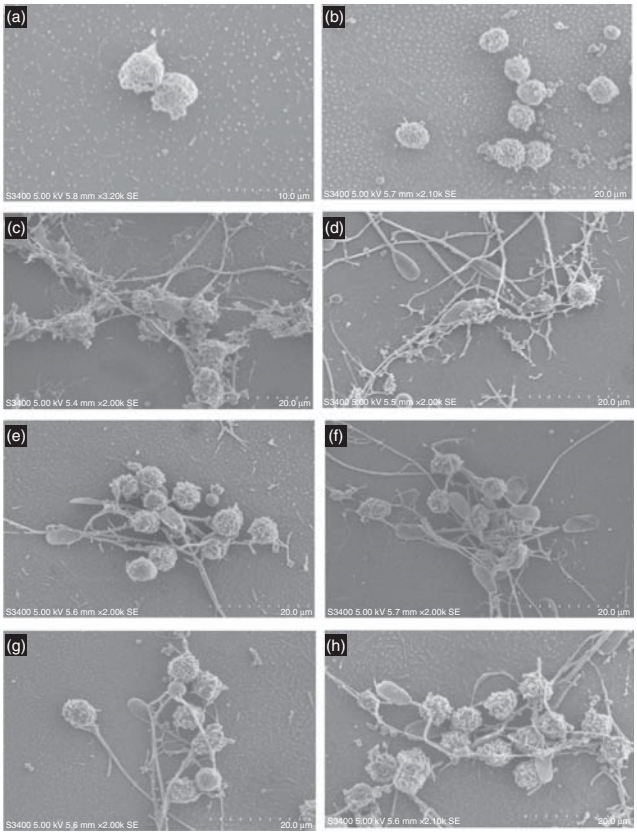

Figure 6 SEM observation of sperm phagocytosis by PMNs. PMNs were incubated with culture medium without any stimulant, with PGE_2 (10^{-7} M), or with LH-stimulated BOEC supernatant for 4 h followed by 1-h phagocytosis assay. Upon the phagocytosis assay, addition of sperm to PMNs induced NETs formation (c and d) compared with that without the addition of sperm (a and b). Furthermore, incubation of PMNs either with PGE_2 (10^{-7} M; e and f) or with LH-stimulated BOEC supernatant (g and h) before the phagocytosis assay resulted in the reduction of NETs formation by PMNs for sperm entanglement.

PGE_2 has been shown to have both pro- and anti-inflammatory effects depending on the activation of the respective PGE_2 receptors (E-prostanoid (EP)), designated as EP_1, EP_2, EP_3, and EP_4 (Hata & Breyer 2004). Signaling through EP_2 and EP_4 mediates the dominant aspects of the PGE_2 anti-inflammatory and suppressive activity (Regan *et al.* 1994, Fujino *et al.* 2005). Thus, PGE_2 has been shown to suppress the functions of human neutrophils via EP_2 receptors (Talpain *et al.* 1995, Burelout *et al.* 2004). The data obtained in the present study show that the EP_2 receptor antagonist abrogated the suppressive effects of both PGE_2 and the LH-stimulated BOEC supernatant on the phagocytic activity of PMNs for sperm. These findings indicate that the mechanism of inhibition of phagocytosis by BOEC-secreted factors partly depends on the PGE_2 interaction with EP_2 receptors on PMNs.

It has been clearly demonstrated that neutrophils either directly phagocytize sperm through cell–cell attachment or entrap them with NETs (Alghamdi & Foster 2005). Accordingly, the factors that suppress sperm phagocytosis by neutrophils may manifest their impact by discouraging both cell–cell attachment and NETs formation. Our results showed that PGE_2 and LH-stimulated BOEC supernatant clearly reduced NETs formation and suppressed direct phagocytosis. Therefore, we think that the reduction of NETs formation led to a decrease in the chances of direct phagocytosis of sperm by PMNs. These observations may account for a better understanding of the mechanism of suppression of sperm phagocytosis. Moreover, PGE_2 has been shown to inhibit the formyl-methionyl-leucyl-phenylalanine-induced phospholipase D (PLD) activity pathway in human neutrophils via EP_2 receptors (Burelout *et al.* 2004). The PLD pathway regulates cell responses such as phagocytosis (Lennartz 1999), secretions, and production of superoxide anions by the NADPH oxidase complex (Liscovitch *et al.* 2000) in human neutrophils. We hypothesize that PGE_2 via EP_2 receptors could affect sperm phagocytosis by neutrophils through the inhibition of the PLD pathway, which in turn changes the phagocytic behavior of PMNs for sperm.

The interaction of bacterial pathogens with human neutrophils results in the induction of phagocytosis of bacteria followed by global changes in PMN gene expression for pro-inflammatory (*TNF* (*TNFα*) and *IL1B* (*IL1β*)) and anti-inflammatory (*IL10*) cytokines (Kobayashi *et al.* 2003). Surprisingly, in our study, the BOEC supernatant suppressed the phagocytic activity of PMNs for sperm without any changes in the gene expression of the above-mentioned cytokines (M A Marey and A Miyamoto 2013, unpublished data). It is speculated that the factors regulating the phagocytosis of sperm by PMNs may be completely different from those involved in the phagocytosis of pathogenic microorganisms. Further investigations are needed to fully understand the possible regulatory mechanisms of phagocytosis of sperm and pathogenic microorganisms by PMNs in the oviduct.

Taken together, the results indicate that during the pre-ovulatory stage sperm are exposed to PMNs in the bovine oviduct and PGE_2 released into the oviduct fluid after LH stimulation may play a major role in the suppression of the phagocytic activity of PMNs for sperm via interaction with EP_2 receptors. Thus, it is concluded that the bovine oviduct provides a PGE_2-rich micro-environment to protect sperm from phagocytosis by PMNs, thereby supporting sperm survival to increase the chances of fertilization.

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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