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Bovine oviduct epithelial cells suppress the phagocytic activity of neutrophils towards sperm but not for bacteria *in vitro*: Immunofluorescence and electron microscopic observations

Mohamed Ali Marey[1,2], Haruhisa Matsukawa[1], Motoki Sasaki[3], Mohamed Aboul Ezz[1,4], Mohamed Samy Yousef[1,5], Ken-ichi Takahashi[6] and Akio Miyamoto[1]

[1]Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, [2]Department of Theriogenology, Faculty of Veterinary Medicine, Damanhur University, Behera, Egypt, [3]Department of Basic Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, [4]Department of Theriogenology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, [5]Department of Theriogenology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt and [6]Genetics Hokkaido Association, Shimizu-cho, Japan

Summary. Previously, we reported that polymorphonuclear neutrophils (PMNs) are constantly existent in the bovine oviduct fluid during the pre-ovulatory stage under physiological conditions. Moreover, incubation of PMNs with bovine oviduct epithelial cells-conditioned medium (BOEC-CM) resulted in suppression of their phagocytic activity for sperm. During pathophysiological conditions, cows may be inseminated by infected semen which exposes oviductal PMNs to allogenic sperm simultaneously with pathogens. This study aimed to visually investigate the role of oviduct epithelium in regulating the phagocytic behavior of PMNs toward sperm as a physiological stimulus, with *Escherichia coli* (*E. coli*) as a pathological stimulus. In our experiment, PMNs were incubated for 2 h in BOEC-CM. Phagocytosis was then assayed by co-incubation of these PMNs either with sperm, *E. coli*, or latex beads. BOEC-CM significantly suppressed the direct phagocytosis of PMNs for sperm, but did not affect their phagocytic activity for *E. coli* or latex beads. Additionally, an investigation with scanning electron microscopy revealed that BOEC-CM suppressed the formation of DNA-based neutrophil extracellular traps (NETs) for sperm entanglement. BOEC-CM did not alter NETs formation towards *E. coli*. A quantification of NETs formation using an immunofluorescence microscopy showed that the areas of NETs formation for *E. coli* were significantly larger than those formed for sperm. Our data clearly show that the bovine oviduct, through secretions, protects sperm from phagocytosis by PMNs and eliminates bacterial dissemination through maintaining the phagocytic activity of PMNs towards bacteria.

Key words: Oviduct, Neutrophils, Sperm, Bacteria, Phagocytosis

Offprint requets to: Akio Miyamoto, PhD, Animal Medical Science Department, Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan. e-mail: akiomiya@obihiro.ac.jp

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Introduction

The oviduct is a highly specialized tube-like structure of the female genital tract that plays important roles in reproduction such as the transport of gametes and embryos, final gametes maturation, fertilization, and early embryo development (Ellington, 1991; Hunter 2012). After insemination in cows, limited numbers (hundreds to thousands) of sperm reach the oviduct where sperm reservoirs are formed (Mitchell et al., 1985; Sostaric et al., 2008) to support sperm capacitation and

viability until ovulation (Wilmut and Hunter 1984; Suarez, 2008). Sperm and embryo possess foreign antigens that can be recognized and attacked by the maternal humoral immunity (Menge, 1969; Gaunt, 1983; Lander et al., 1990). Moreover, the oviduct may be infected by microbes (ascending from uterus or descending from abdominal cavity) which induce inflammation of the oviduct, and disruption of oviductal functions (Sheldon et al., 2010). Therefore, the oviduct should be equipped with a well-developed immune system that protects it from infection simultaneously with maintaining a proper microenvironment for survival of sperm, oocyte and early embryo.

The innate immune system is the first line of host defense against pathogens, and PMNs are the most abundant leukocytes involved in this response (Tapper, 1996). Recently, we conducted a flow cytometrical analysis to investigate the existence of PMNs in bovine oviduct fluid. PMNs were existent in the oviduct fluid during the pre-ovulatory stage in stable numbers (average 3-5×10^3 cells /oviduct flush), constituting around 12-17% of the total leukocytes population (Marey et al., 2014). Later on, we found that PMNs are constantly existent in the oviduct fluid throughout the estrous cycle (Marey and Miyamoto, unpublished data). PMNs exert host defense functions either by phagocytosis, or by the release of proteolytic enzymes and the generation of oxygen-derived reactive agents (Tapper, 1996). PMNs phagocytize pathogens either directly through cell-cell attachment or entrap them with NETs, which are strand-like webs of decondensed chromatin, which also contain granule proteins (e.g., histones) that ensnare and kill microbes (Brinkmann et al., 2004). The formation of NETs (NETosis) can be triggered by microbial infections, such as bacteria, fungi and parasites (Kaplan and Radic, 2012). Additionally, sperm can trigger PMNs to release NETs for sperm entanglement (Alghamdi and Foster, 2005).

Previously, we have shown that incubation of PMNs with the BOEC-CM (stimulated either with or without luteinizing hormone (LH)) resulted in suppression of their phagocytic activity for sperm. Additionally, LH-stimulated BOEC-CM completely blocked NETs formation for sperm entanglement (Marey et al., 2014). Concurrently with sperm, PMNs may be exposed to pathogens that may accompany sperm to the oviduct milieu. We hypothesized that the local immune system of the oviduct can efficiently differentiate between the allogenic sperm and pathogens. Therefore, we conducted this *in vitro* experiment to investigate whether the suppressive effect BOEC-CM on the phagocytic behavior of PMNs is restricted to sperm only or extends to involve bacteria, i.e. *E. coli*.

Material and methods

Ethics statement

All experiments described in this article were conducted in accordance with the guiding principles for the care and use of research animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan. The protocol was approved by the Committee of Ethics of Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine (Permit number 27-74).

Primary culture of BOECs

Isolation and culture of BOECs were based on the method described previously (Wijayagunawardane et al., 1999; Marey et al., 2014) with minor modifications. Briefly, oviducts were collected from the local slaughterhouse (Hokkaido Livestock Co., Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan). In the laboratory, BOECs were mechanically dislodged, purified, and cultured in D-MEM/F12 (gibco, USA; supplemented with 2.2% $NaHCO_3$, 0.1% gentamicin, 1% amphotericin, and 10% fetal calf serum; FCS; Bio Whittaker, Walkersville, MD) in 6-well culture dishes (Nalge Nunc International, Roskilde, Denmark) at 38.5°C in 5% CO_2 and 95% air until monolayer formation. After monolayer formation, the cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH), re-plated in 6-well culture dishes at a density of 3×10^4 cells/ml, and cultured until sub-confluence. The growing BOEC monolayer was then cultured in medium supplemented with 0.1% FCS and incubated for 24 h. Finally, the culture medium was collected and stored at -80°C until used. The cells were collected and cell viability was tested using Trypan-blue staining and confirmed to be more than 95%. The purity of BOECs was confirmed by anti-cytokeratin-(CK1) immunostaining, and by characteristic epithelial morphology.

Preparation of PMNs

PMNs were isolated as previously described (Jiemtaweeboon et al., 2011). Blood collection experiments were conducted at the field center of animal science and agriculture of Obihiro University. Briefly, PMNs were separated using Ficoll-paque solution (Lymphoprep, Axis Shield, Oslo, Norway). The purity of PMNs as evaluated by flow cytometry was >98%, and the viability was around 99% as assessed by Trypan blue staining. Prior to phagocytosis assay, PMNs were suspended at a density of 1×10^7 cells/ml with BOEC-CM in culture tube and incubated for 2 h. BOEC-CM was obtained by centrifugation of BOEC culture medium at 1000×g for 10 min to remove any cells or debris. The upper 70-80% of supernatant was used for PMNs culture. After PMNs incubation, PMNs were washed 2 times by phosphate-buffered saline without calcium or magnesium ($PBS^{-/-}$) and used for phagocytosis assay.

Preparation of sperm

Concurrently with PMNs preparation, sperm were

prepared as previously reported by Marey et al. (2014). *In vitro* capacitation of bull sperm was induced by using modified Tyrode's albumin, lactate, and pyruvate medium (Sp-TALP). Briefly, 1 h swim-up was carried out to obtain highly active and motile sperm, and followed by 4 h incubation in Sp-TALP medium supplemented with 10 μg/ml heparin. Capacitation was confirmed by induction of acrosome reactions by using 100 μg/ml lysophosphatidylcholine for 15 min. Afterwards, sperm were washed and suspended in Tyrode's medium containing lactate, pyruvate, and HEPES (TL-HEPES), and then were used for phagocytosis assay.

Phagocytosis assay

Phagocytosis assay was performed according to Matthijs et al. (2000) and Marey et al. (2014) with minor modifications. Briefly, the 2 h incubated PMNs were suspended in TL-HEPES. Equal volumes of PMNs suspension (3×10^6 cells/ml) were mixed either with sperm suspension (6×10^6 cells/ml), latex beads, carboxylate modified polystyrene, fluorescent yellow green (Sigma-Aldrich, Tokyo, Japan) (12×10^6 solids/ml), or fluorescently labelled *E. coli* (Thermo Fisher Scientific, Denmark) (1×10^6, 6×10^6, or 12×10^6 particles/ml), in a 96-well untreated polystyrene microtest plate (Thermo Scientific, Denmark). Fresh serum 12% (v/v) was added to this suspension and the final volume of the whole suspension was 100 μl and incubated for 60 min with gentle swirling on a test-plate shaker. After incubation, an equal volume of heparin (40 mg/mL in TL-HEPES) was added to allow 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 fluorescence microscopy at ×400 magnification connected to a digital camera and a computer system (Leica Application Suite, LAS, Germany). Minimally, 400 PMNs were counted in different areas of specimen. The percentage of PMNs with phagocytized sperm was considered as phagocytosis rate. Quantification of the number of phagocytizing PMNs was performed independently by 2 different observers. Phagocytosis ratios for *E. coli* or latex beads were counted manually like sperm under the fluorescence microscope and confirmed by using flow cytometry. For flow cytometry evaluation, 20 µl of the leukocyte suspension was diluted in Macs separation buffer (MACS Miltenyi Biotec, Tokyo, Japan) and analyzed by flow cytometry (Beckman Coulter, Inc., CA, UAS).

Scanning electron microscopy (SEM)

PMNs phagocytize pathogens or sperm either directly through cell-cell attachment or entrap them with NETs and the latter is mainly detected by SEM (Brinkmann et al., 2004; Alghamdi and Foster, 2005). Therefore, SEM was used to investigate the effect of BOEC-CM on NETs formation by PMNs towards sperm, *E. coli*, or latex beads. Neutrophils (1×10^7 cells/ml) were incubated in culture medium without any stimulus, or with BOEC-CM for 2 h, and phagocytosis was then assayed. SEM was conducted as previously reported by Marey et al. (2014).

Immunofluorescence microscopy (NETs fluorescence)

After 1 h of co-culturing of PMNs with sperm, *E. coli*, or latex beads, cells were stained with 30 nM 4',6-diamidino-2-phenylindole (DAPI blue, Life Technologies) to target DNA. DAPI was used to visualize DNA of NETs. DAPI stains condensed chromate in bright blue with increased intensity proportional to chromatin condensation, making it an efficient nuclear stain (Mascetti et al., 2001). Cell images were taken with fluorescence microscope (Leica Application Suite, LAS, Germany), and NETs were quantified based on the area of DNA per number of cells detected by DAPI. ImageJ software version series IJ 1.46r (National Institute of Health, USA) was used for NET quantification.

Statistical analysis

Data are presented as the mean ± SEM of 3 independent experiments. Statistical analyses were performed with IBM® SPSS® Statistics 24 (IBM Corp., Armonk, NY, USA). Statistical significance between two groups was made using student's t-test and all results were considered to be statistically significant at $P < 0.05$. In all figures, significant differences were illustrated with asterisks (*p<0.05; **p<0.01).

Results

Effect of BOEC-CM on the phagocytic activity of PMNs for sperm, latex beads, and E. coli

Previously we investigated the suppressive effect of BOEC-CM on the phagocytic activity of PMNs toward different sperm ratios (the PMN: sperm ratio was 2:1, 1:1, or 1:2, Liu et al., 2014; Marey et al., 2014, 2016a) respectively. In this study, to clearly illustrate BOEC-CM suppressive effect on PMN phagocytosis, the PMNs: sperm ratio was fixed at 1:2 (PMNs suspension (3×10^6 cells/ml) and sperm suspension (6×10^6 cells/ml). However, in a preliminary experiment, several concentrations of latex beads were tested, and only latex beads at (12×10^6 solids/ml) induced phagocytosis ratios close to those induced by the used sperm suspension, average 42% for latex beads, and 43% for sperm. Moreover, our results showed that *E. coli* dose-dependently stimulated the phagocytic activity of PMNs. Incubation of PMNs for 2 h in BOEC-CM prior to phagocytosis assay resulted in significant suppression of their phagocytic activity for sperm treated to induce capacitation (Fig. 1A). Meanwhile, 2 h incubation of

PMNs in BOEC-CM did not induce any significant differences in the phagocytic activity of PMNs for latex beads (Fig. 1B,D) or *E. coli* (Fig. 1C,E) compared to control.

NETs formation observed by SEM

During phagocytosis assay, the addition of sperm to PMNs induced NETs formation (Fig. 2A,B). Meanwhile, incubation of PMNs with BOEC-CM prior to phagocytosis assay suppressed NETs formation by PMNs for sperm entanglement (Fig. 2C,D). Additionally, the addition of *E. coli* particles to PMNs induced NETs formation (Fig. 2E,F). Moreover, incubation of PMNs with BOEC-CM prior to phagocytosis assay did not alter NETs formation by PMNs for *E. coli* (Fig. 2G,H). Furthermore, the addition of latex beads failed to stimulate NETs formation by PMNs pre-incubated either without (Fig. 2I,J) or with BOEC-CM (Fig. 2K,L).

Immunofluorescence of NETs formation

In all conducted phagocytosis assays, the total PMNs numbers were kept equal. Therefore, the areas of decondensed chromatin per total number of cells were stained by DAPI, visualized by fluorescence microscope, and measured to quantify NETs. Incubation of PMNs either with sperm (Fig. 3A,B), or *E. coli* (Fig. 3C,D) induced NETs formation. Meanwhile, latex beads failed to stimulate PMNs to release NETs (Fig. 3E,F). Quantification of NETs formation revealed significant differences ($P<0.01$) in DNA-histone area per number of PMNs triggered by *E. coli* or sperm. Thus, the quantities

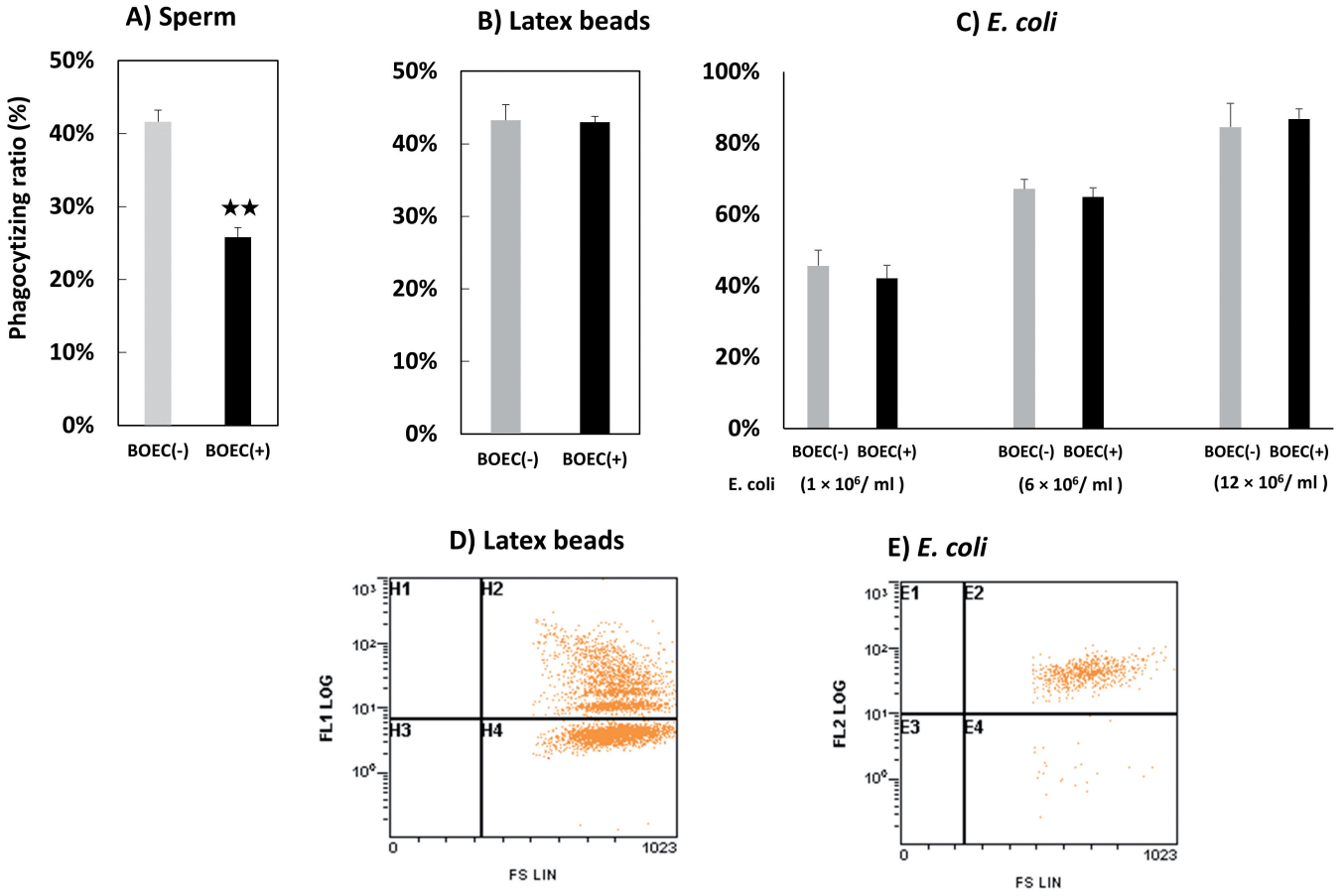

Fig. 1. Percentage of *in vitro* phagocytosis of polymorphonuclear neutrophils (PMNs) for sperm **(A)**, latex beads **(B)**, and *E. coli* **(C)**. PMNs (3×10^6 cells/ml) were incubated with culture medium without any stimulant, or with bovine oviduct epithelial cell-conditioned medium (BOEC-CM) for 2 h followed by 1 h phagocytosis assay with sperm (6×10^6 sperm/ml), latex beads (12×10^6 solids/ml), or *E. coli* (1×10^6, 6×10^6, or 12×10^6 particles/ml). Numerical values are presented as the mean ± SEM of 3 experiments. Significant differences between the treatments were illustrated with asterisks (*$P<0.05$; **$P<0.01$). **D and E.** Representative flow cytometrical analysis of PMNs phagocytosis for latex beads and *E. coli*. After phagocytosis assay, a total of 5,000 PMNs were counted and depicted according to their relative size, density and granularities as forward scatter (FS) and log green fluorescence (FL) cytogram. The narrow regions (H2) or (E2) represent the ratio of PMNs that phagocytized latex beads or *E. coli* and exhibited green fluorescence (42.89% versus 96.05%) in Fig. 1. D, and E respectively. Pre-incubation of PMNs with BOEC-CM prior to phagocytosis assay did not show any significant differences compared to control group in both assays. Graphs are representative of three independent experiments.

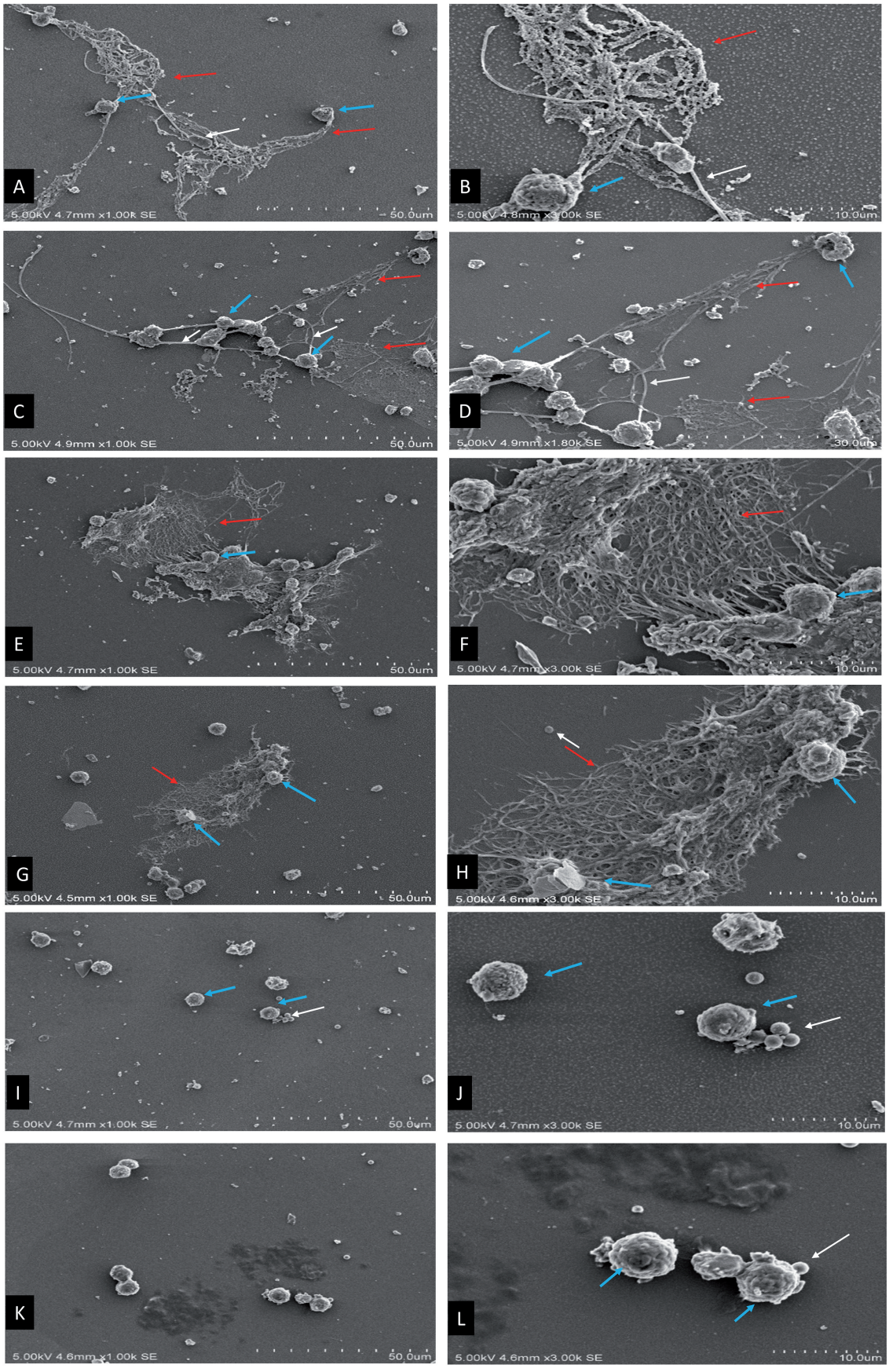

Fig. 2. Scanning electron microscopic observation for polymorphonuclear neutrophils (PMNs) phagocytosis for sperm, *E. coli*, or latex beads. PMNs were incubated with culture medium without any stimulant, or with bovine oviduct epithelial cell-conditioned medium (BOEC-CM) for 2 h followed by 1 h phagocytosis assay with sperm, *E. coli*, or latex beads. Upon phagocytosis assay, addition of sperm to PMNs induced neutrophil extracellular traps (NETs) formation **(A and B).** Meanwhile, incubation of PMNs with BOEC-CM prior to phagocytosis assay suppressed NETs formation by PMNs for sperm entanglement **(C and D)**. Addition of *E. coli* to PMNs induced neutrophil extracellular traps (NETs) formation **(E and F)**. Moreover, incubation of PMNs with BOEC-CM prior to phagocytosis assay did not affect NETs formation by PMNs for *E. coli* entanglement **(G and H)**. Furthermore, addition of latex beads to PMNs pre-treated with **(I and J)** or without **(K and L)** BOEC-CM did not stimulate PMNs to release neutrophil extracellular traps (NETs). White arrows refer to sperm, *E. coli*, or latex beads, blue arrows refer to PMNs, and red arrows refer to NETs. Micrographs are representative of three independent experiments.

of *E. coli*- mediated NETosis were significantly larger than those for sperm-mediated NETosis (Fig. 4).

Discussion

The present study, using *in vitro* assays, provides visual evidence for the ability of the oviduct epithelium to control the phagocytic behavior of PMNs towards sperm and pathogen. Our results clearly showed that BOEC-CM suppressed PMNs phagocytosis towards sperm, while maintaining their phagocytic activity against *E. coli*.

PMNs are constantly existent in the bovine oviductal fluid during the pre-ovulatory stage of the estrous cycle. Thus, upon their arrival to the oviduct, sperm may be attacked by PMNs floating in the oviduct fluid. Interestingly, incubation of PMNs with BOEC-CM weakened their phagocytic activity towards sperm. SEM observations revealed that the LH-stimulated BOEC-CM prevented the formation of NETs leading to protection of sperm from being trapped and degraded by PMNs (Marey et al., 2014). Whilst the former study focused on the interaction between innate immune cells, PMNs, and sperm in the bovine oviduct, we could not ignore the probability that the oviduct might be impregnated by pathogens after insemination. Therefore, it was essential to investigate the possible role of oviductal epithelium, in adjusting the phagocytic behavior of PMNs to the various antigens to which they were exposed. Here, we visually investigated the potential differences in the response of PMNs to sperm as physiological antigens, and *E. coli* as pathological antigens as well as latex beads.

Initially, our results showed that either *E. coli*, sperm or latex beads stimulated the direct phagocytic behavior of PMNs but with different ratios. *E. coli* was the

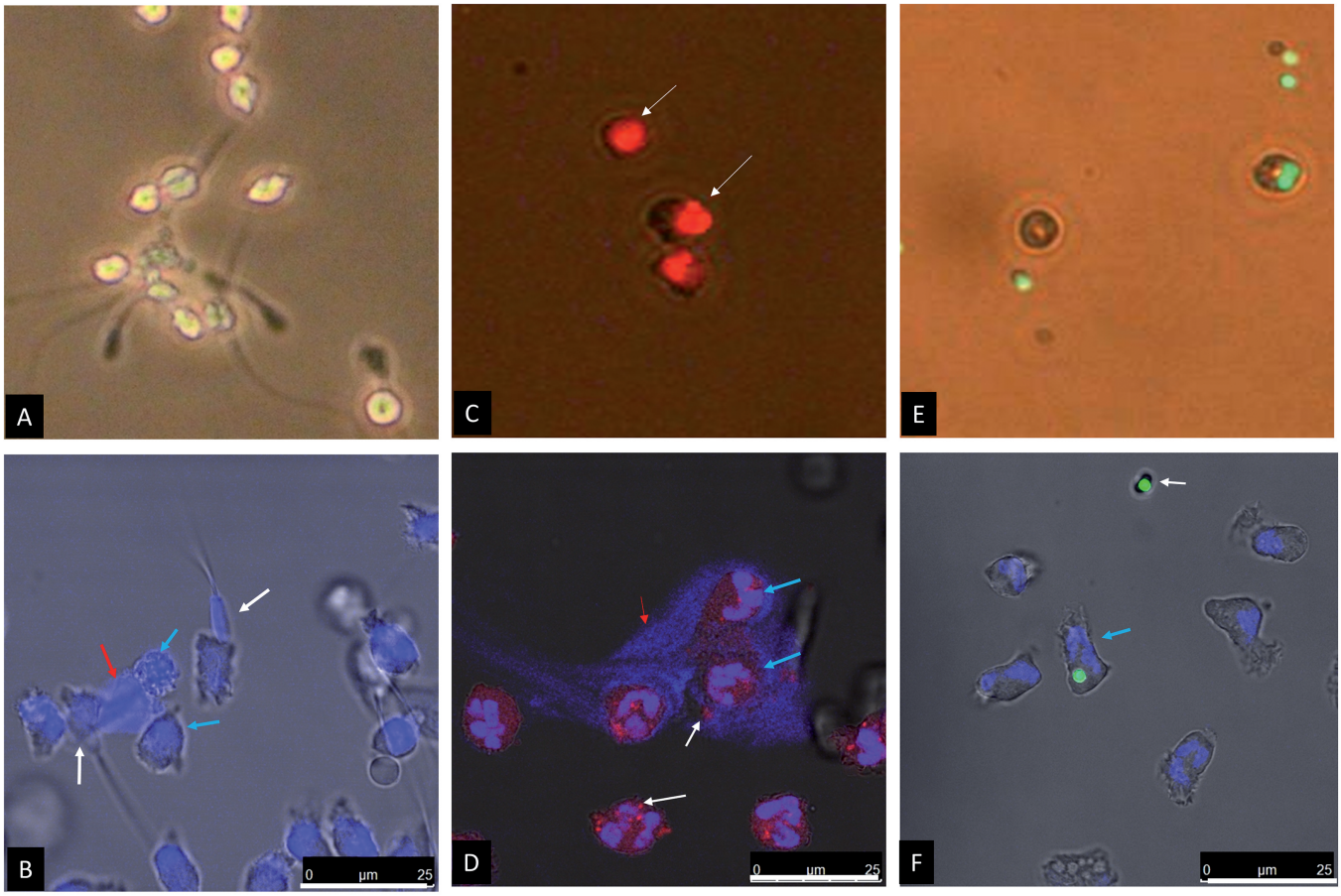

Fig. 3. Representative immunofluorescence scanning for polymorphonuclear neutrophils (PMNs) phagocytosis for sperm, *E. coli*, and latex beads. PMNs were incubated with sperm, *E. coli*, or latex beads for 1 h as phagocytosis assay. Immunofluorescence staining was performed to detect NETs formation which is depicted by DAPI staining. Addition of sperm to PMNs induced direct cell-cell attachment **(A)** and neutrophil extracellular traps (NETs) formation **(B)**. Also, addition of *E. coli* to PMNs induced direct cell-cell attachment **(C)** and neutrophil extracellular traps (NETs) formation **(D)**. Meanwhile, addition of latex beads to PMNs induced direct cell-cell attachment **(E)** and but did not induce neutrophil extracellular traps (NETs) formation **(F)**. White arrows refer to sperm, *E. coli*, or latex beads, Blue arrows refer to PMNs and red arrows refer to NETs. Micrographs are representative of three independent experiments.

strongest stimulus for PMNs direct phagocytic activity. Moreover, treatment of PMNs with BOEC-CM disrupted their direct phagocytic activity towards sperm, but not for *E. coli* nor latex beads. NETs formation was observed using SEM. Importantly, both sperm and *E. coli* stimulated NETs formation. Meanwhile latex beads failed to trigger PMNs for unleashing NETosis. Additionally, pre-incubation of PMNs in BOEC-CM weakened the NETs formation for sperm but did not affect NETs formation for *E. coli*.

Previously we investigated the factors that regulate PMNs phagocytosis towards sperm in oviduct. In this regard, it was found that LH stimulated the release of prostaglandin E_2 (PGE_2) from BOECs which subsequently acted, *via* its receptor (EP_2), to suppress both direct cell-cell attachment and NETs formation by PMNs towards sperm (Marey et al., 2014). Later on, more focus was given to the inhibitory effect of PGE_2 on NETs formation. It was found that PGE_2 inhibits NETosis by activation of cyclic adenosine monophosphate-protein kinase A dependent pathway through the activation of its receptors, EP_2 and EP_4 (Domingo-Gonzalez et al., 2016; Shishikura et al., 2016). In addition to prostaglandins, BOECs secrete vasoactive peptides, angiotensin II and endothelin-1, for regulation of the oviductal contractility (Wijayagunawardane et al., 2001a,b). Of note, angiotensin II upregulated the phagocytic activity of PMNs towards sperm (Marey et al., 2016a), while endothelin-1 downregulated it (Marey et al., 2016b). These findings suggest an emerging role of prostaglandins-endothelin-angiotensin system in the regulation of sperm survivability in the bovine oviduct (Marey et al., 2016c).

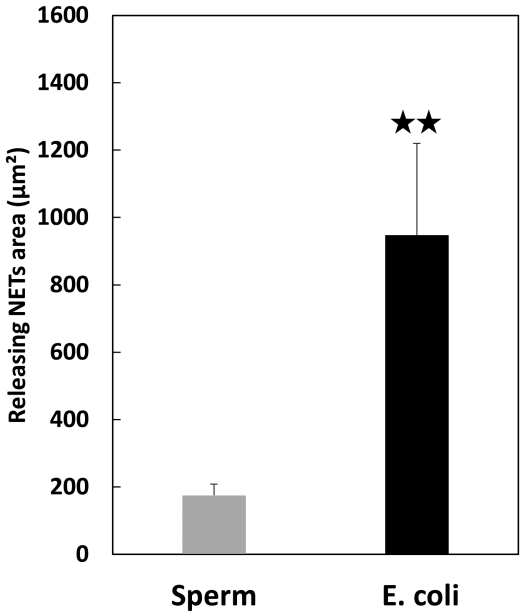

Fig. 4. Quantification of NETs formation by PMNs towards sperm and *E. coli*. PMNs were incubated with sperm, or *E. coli* f or 1 h as phagocytosis assay. Immunofluorescence staining was performed to detect NETs formation which is depicted by DAPI staining. NET formation was quantified by ratio of DNA-histone area per number of PMNs triggered by *E. coli* or sperm. Asterisk ** denotes a significant variance (P<0.01). Data are presented as mean ± SEM of three independent experiments.

The oviduct is the main site for sperm capacitation in mammals (Hunter, 2012). Therefore, throughout the whole experiments, we treated sperm with heparin to induce capacitation. However, sperm susceptibility to phagocytosis may partially depend on the physiological status of sperm. Thus, it has been reported that intact boar sperm are better phagocytized than damaged or dead ones. Additionally, treatment of the boar sperm to induce capacitation *in vitro* considerably reduced the phagocytosis of spermatozoa by PMNs (Matthijs et al., 2000). Moreover, in human, post-capacitated spermatozoa were more preferentially phagocytized by macrophages and this effect was not related to vitality, motility, or the acrosomal status of sperm (Oren-Benaroya et al., 2007). Further studies are needed to investigate the effect of BOEC conditioned medium on PMNs phagocytosis for different forms of bovine sperm.

Sperm-oviduct interaction can induce several dynamic changes in the immunological microenvironment of oviduct. *In vitro* BOEC-sperm binding stimulates the gene expression for the anti-inflammatory cytokines (IL-10 and TGFβ-1) and slightly decreased the pro-inflammatory cytokines (TNFα and IL-1β) in BOECs (Yousef et al., 2016). Moreover, BOEC-CM after BOEC-sperm binding induced mRNA expressions of the anti-inflammatory cytokines (IL-10 and TGFβ-1) and decreased the pro-inflammatory cytokines, TNFα, in PMNs (Yousef et al., 2016). All these findings support the hypothesis that sperm communicate with oviductal epithelium to induce their own protection from the maternal immune response and elicit a state of immune tolerance against these allogeneic paternal cells.

Lipopolysaccharide (LPS), a major component of the outer membrane of *E. coli*, is reported as a strong stimulus for direct cell-cell attack as well as NETs formation by PMNs (Brinkmann et al., 2004). Moreover, LPS stimulates neutrophils mainly via Toll- like receptor-4 (TLR-4) signaling pathway to produce inflammatory mediators including IL-1β, IL-12, IFN-γ, IL-8, and TNF-α (Sohn et al., 2007; Worku and Morris, 2009). We have shown that LPS can change the local oviductal milieu into a proinflammatory conditions, where it stimulates the gene expression of TLR-4, COX-2, NFKBIA, IL-1β, and TNF-α in BOECs *in vitro* (Kowsar et al., 2013). These above-mentioned findings may partially explain our current observations for the inability of BOEC-CM to alter the direct phagocytic activity of PMNs towards *E. coli* compared to control. It seems that the stimulatory effect of LPS on the phagocytic activity of PMNs towards *E. coli* overrides the suppressive effect of the local metabolites existent in BOEC-CM in our *in vitro* assay.

Nevertheless, it is still incompletely understood how pathogens induce signaling events that result in NETosis, the model of NETosis differs according to type of pathogen (Delgado-Rizo et al., 2017). Therefore, to visually understand the potential differences in PMNs NETosis for sperm and *E. coli*, immunofluorescence microscopy was used to quantify NETs formed towards sperm and *E. coli*. Immunofluorescence visualization of NETs showed clearly larger quantities of NETs formed for *E. coli* compared to those formed for sperm. Moreover, SEM observations showed denser and thicker NETosis formed for sperm compared to those for *E. coli*. This suggests that the nature of NETs formed towards sperm completely varies from those formed for *E. coli*. Previously, it has been demonstrated that the initiation and evasion of NETs depend mainly on the type of pathogen and their metabolic byproducts as a stimulus for unleashing NETs (Delgado-Rizo et al., 2017). Moreover, PMNs can clearly discriminate between LPS derived from different pathogens and strains, and selectively release NETs (Pieterse et al., 2016). Further investigations are needed to fully understand the differences in the detailed molecular processes and sequential steps of NETs formation towards sperm and pathogens.

Altogether, our results provide visual clarifications that the bovine oviduct has a highly specialized local immune system that can differentially recognize and therefore efficiently respond to different arrays of foreign entities to which it is exposed.

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

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