Sperm enter glands of preovulatory bovine endometrial explants and initiate inflammation

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Abstract

We previously reported that sperm binding to cultured monolayers of bovine uterine epithelial cells induces an acute inflammatory response involving the Toll-like receptor (TLR2) signaling pathway. This response serves to clear the uterus of sperm and thereby prepares the endometrium for implantation. The endometrium is lined by surface epithelial cells; however, epithelial cells also line uterine glands. To investigate the source of the immune response, we used an explant model. Explants of bovine endometrium were incubated with bull sperm illuminated by JC1 fluorescent labeling in their mitochondria. The sperm glided over the surface epithelium until they encountered and entered uterine glands, where they remained. Scanning electron microscopy of explants revealed polymorphonuclear neutrophils (PMNs) in uterine glands along with sperm. In the absence of sperm, PMNs were not seen in glands. The incubation of sperm with explants resulted in an acute inflammatory response, seen as the upregulation of mRNA expression of *IL8*, *TNFA*, *IL1B*, *PGES* and *TLR2* in whole explants, as well as increased TNFA protein expression in uterine glands. TLR1/2 antagonist reduced sperm numbers in the glands and inhibited the increase of *TNFA*. Our observations suggest that uterine glands serve as a site where sperm interact with the uterine epithelium to trigger the innate immune response to clear excess sperm from the uterus.

Reproduction (2020) **159** 181–192

Introduction

In the course of migrating through the female tract to the egg, mammalian sperm interact closely with the epithelium lining the tract (Suarez 2016, Dutta *et al.* 2019). Most work in the past 20 years has focused on the mammalian sperm interaction with the oviductal epithelium, which is important because the oviduct is the site of fertilization and the most common site of sperm storage (Suarez *et al.* 1997, Suarez 2002); however, the uterus also plays an important role in its interactions with sperm. That is, the uterus must clear sperm from its lumen as part of the preparation of uterus to receive embryos (Mattner 1968, Mitchell *et al.* 1985).

The removal of excess, defective and dead sperm from the uterus begins soon after insemination. In the cow, many sperm are removed rapidly after semen deposition through fluid backflow (Dobrowolski & Hafez 1970, Suga & Higaki 1971). In addition, the bovine uterus has a well-developed innate immune system that can mount an inflammatory response to sperm (Fair 2015) as well

as to pathogens (Sheldon *et al.* 2019). The inflammatory response to sperm contributes to their rapid removal, which not only prepares the endometrium to receive an embryo but also prevents the development of acquired immune responses against sperm, that is, the production of anti-sperm antibodies that could reduce the fertility of the female (Zralý *et al.* 2003, Hansen 2011).

Bull sperm are rapidly removed during the inflammatory response mainly by polymorphonuclear neutrophils (PMNs) (Mattner 1968). The PMN influx after semen deposition is essential not only to remove sperm but also to remove pathogens that may be introduced during mating (Mattner 1968, Alghamdi *et al.* 2009). Influx of PMNs into the uterine lumen to remove sperm and pathogens has also been reported in horses (Katila 1995) and, pigs (Kaeoket *et al.* 2003). The elimination of sperm in the uterus by PMNs is mediated by two processes: phagocytosis of sperm and the formation of DNA-based neutrophil extracellular traps (Alghamdi *et al.* 2009, Hong *et al.* 2017).

We recently reported that sperm binding to monolayer cultures of bovine uterine epithelial cells (BUECs) induces an acute inflammatory response in the epithelium (Elweza *et al.* 2018) by Toll-like receptor (TLR) signaling pathway via TLR2 (Ezz *et al.* 2019). However, *in vivo*, sperm interactions with the immunological defenses of the uterus could be mediated by the surface epithelium and/or glandular epithelium. Therefore, we adapted a bovine explant culture model (Borges *et al.* 2012) to investigate the site and dynamics of sperm interactions with the uterine epithelium under conditions that more closely resemble those *in vivo*. Our initial observations prompted us to hypothesize that sperm interact with the glandular epithelium of the preovulatory uterus to trigger immune responses. To test this hypothesis, we identified the site of sperm interactions using fluorescence video microscopy and scanning electron microscopy (SEM). Further, we evaluated the involvement of TLR2 in the sperm–uterine interaction and the inflammatory processes.

Materials and methods

All experiments described in this article were conducted in accordance with the relevant guidelines and regulation principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan. The Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine approved the protocol (Permit number 27-74).

Reagents and media

Chemicals were purchased from Wako Pure Chemical Industries, unless otherwise stated. A modified Tyrode balanced salt solution, Tyrode albumin lactate pyruvate (TALP) (Parrish *et al.* 1988), was used for washing and diluting sperm and for incubating endometrial explants with sperm. TALP consisted of 99 mM NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 0.39 mM NaH₂PO₄, 10 mM HEPES-free acid, 2 mM CaCl₂, 1.1 mM MgCl₂, 25.4 mM sodium lactate, 0.11 mg/ml sodium pyruvate, 5 µg/ml gentamycin and 6 mg/ml BSA pH 7.4. TALP was equilibrated in a 38.5°C incubator (bovine core body temperature) with 5% CO₂ in humidified air before use. Saline was used as a transport medium for uterine horns and consisted of 154 mM NaCl, 10 µg/mL penicillin–streptomycin (Gibco) and 10 µg/mL amphotericin B (Gibco) pH 7.4.

Animals and tissue preparation

Uterine samples of cows were collected from the local slaughterhouse (Hokkaido Livestock, Doto Plant Tokachi Factory, Obihiro, Hokkaido, Japan). The reproductive tracts were trimmed free of surrounding tissues, opened and macroscopically examined to be free from pus, inflammation and abnormal color. The phase of the estrous cycle was identified based on the appearance, weight and color of the

corpus luteum and the follicular diameter according to a protocol described previously (Wijayagunawardane *et al.* 1998). The ipsilateral horns relative to the ovary containing the mature follicle of the preovulatory phase (days 19–22) and those relative to the ovary containing the acute corpus luteum of the luteal phase (days 10–12) were isolated and transported on ice-cold saline to the laboratory. Then, the uterine horns were incised longitudinally. Endometrial explants were prepared according to a method described previously (Borges *et al.* 2012) with modifications. Using an 8 mm biopsy punch (Kai industries, Oyana, Japan), disks of endometrial tissue were dissected from the glandular (intercaruncular) endometrial regions (Atkinson *et al.* 1984). Then, surgical scissors were used to dissect approximately 2 mm intact endometrium from the underlying tissue. Explants were immediately placed, epithelial surface up, in a glass petri dish filled with TALP and kept at 38.5°C by a temperature-controlled micro warm plate (KM-2, Kitazato, Japan). A coating of clear mucus could be seen on the surface epithelium of the explants. The explants were incubated in a 38.5°C incubator with 5% CO₂ in humidified air for 15 min before experiments were initiated. Normal tissue architecture and the viability of the explants were confirmed using hematoxylin and eosin (HE) tissue sections (Borges *et al.* 2012) and Caspase 3 mRNA expression (Zhang *et al.* 2018).

Sperm sample preparation

Fresh semen samples of highly fertile Holstein bulls were obtained from Genetics Hokkaido Association, Hokkaido, Japan. Fresh semen was immediately diluted in egg-yolk extender (1:1) in a 15 ml tube. The tube was maintained in a temperature-controlled heat jacket (15°C) during transport to the laboratory (1 h). Then the semen was maintained in 15°C until the beginning of the experiment (6 h). The choice of storage of extended semen at 15°C for transport was based on reports by Murphy *et al.* (2015) and testing at 4°C and 22°C. Immediately prior to the experiment, sperm were washed by diluting 2 mL extended semen with 3 mL TALP, centrifuging at 170g for 10 min (25°C), resuspending the pellet in 5 mL TALP and repeating the centrifugation twice. The resulting sperm pellet was resuspended in TALP. Sperm concentration was determined using a hemacytometer (C-chip, NanoEnTek, Korea) and adjusted to 10⁶ sperm/mL for incubation with endometrial explants. Heat-inactivated sperm were prepared by the incubation of washed fresh sperm in a water bath at 56°C for 30 min (Kodithuwakku *et al.* 2007).

Incubation of sperm with bovine endometrial explants

To investigate the time-dependent response of endometrial explants to sperm, preovulatory phase explants were incubated with 10⁶ sperm/mL for 0, 0.5 and 2 h in a 38.5°C incubator with 5% CO₂ in humidified air using 24 well plates with 0.5 mL TALP/well. This sperm concentration had previously been shown to stimulate innate immune responses in monolayer cultures of BUECs (Elweza *et al.* 2018, Ezz *et al.* 2019). Explants without sperm served as controls for each time point. To investigate the effect of heat-inactivated sperm, the preovulatory phase explants were incubated with 10⁶ heat-inactivated sperm/mL for 2 h. To

investigate the effect of sperm, the luteal phase explants were incubated with 10^6 sperm/mL for 2 h. Explants without sperm served as controls. The involvement of the TLR2 in sperm–uterine attachment and the inflammatory process was examined using TLR1/2 antagonist-CU-CPT22 (Merck) (Cheng *et al.* 2012). The preovulatory phase explants were pre-incubated with 1 μ M antagonist in a 38.5°C incubator with 5% CO₂ in humidified air for 30 min. Then the explants were incubated with 10^6 sperm/mL for 2 h. Explants without antagonist and sperm served as controls. All experiments were repeated five times using endometrial explants from five different uteri (three wells per treatment per experiment). Explants were collected and washed three times in TALP and stored in TRIZOL (Invitrogen), at –80°C until total RNA extraction.

JC1 labeling of sperm

To see the sperm on the epithelial surface of explants, the sperm were stained with the mitochondrial stain JC1 (AdipoGen, San Diego, CA, USA). For this approach, 0.5 mL 10^6 sperm/mL were pre-incubated with 6.4 μ M JC1 in a 38.5°C incubator with 5% CO₂ in humidified air for 15 min, directly before adding them to explants. It was not necessary to wash labeled sperm before adding them to explants, because the dilution of the JC1 dye in the explant culture medium provided us with helpful faint images of the epithelial surfaces without obscuring the view of sperm.

Observation of sperm behavior after incubation with endometrial explants

To visualize the behavior of sperm on endometrial explants, JC1-labeled sperm were incubated with explants for 5 min and 30 min. To evaluate the effect of TLR1/2 antagonist on sperm behavior, the preovulatory phase endometrial explants were pre-incubated with 1 μ M TLR1/2 antagonist in a 38.5°C incubator with 5% CO₂ in humidified air for 30 min. Then JC1-labeled sperm were incubated with pre-incubated explants for 30 min. Explants without antagonist served as controls. Endometrial explants were oriented as epithelial surface uppermost. In order to see the sperm, each explant was rinsed through wells containing 38.5°C TALP and transferred to a well that contained 0.5 mL 38.5°C TALP; the explants were oriented as epithelial surface downward on the heated stage of the inverted microscope. Sperm behavior in endometrial explants was viewed using an all-in-one fluorescence microscope (Keyence, BZ-X800, Osaka, Japan) equipped with a temperature-controlled thermal plate (TPi-SQX, Tokai Hit, Japan). Videos and images were taken using the BZ-X TexasRed (OP-87765) and BZ-X GFP (OP-87763) filters set for red and green wavelengths respectively. During the video recording, the focus was adjusted to count and visualize all sperm that entered and remained in the glands and sperm on the surface epithelium.

Determination of the numbers of sperm in uterine glands

Videos (JC1-labeled sperm) recorded at 30 min of explants with sperm±antagonist were assessed for numbers of sperm

in uterine glands. Recorded videos were converted to gray scale, and contrast was optimized in the same way on all videos using ImageJ (Version 1.51j8) before counting sperm. Counts were made of three glands of equal size in each experimental treatment.

Histology and immunohistochemistry

To investigate the effect of sperm on the preovulatory phase endometrium, the explants were incubated with 10^6 sperm/mL for 4 h. Explants without sperm served as controls. The experiment was repeated four times using endometrial explants from four different uteri. Explants were gently rinsed in TALP and fixed in 10% formalin in phosphate buffer (PB) (pH 7.4). After 24 h, the fixed tissue samples were transferred to 70% ethanol and then dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin and cut serially in 4 μ m thick slices. The endometrial sections were placed on an aminopropyl-triethoxy-silane-coated slides (S8226, Matsunami Glass Int., Osaka, Japan) and deparaffinized. The sections were stained with HE for the observation of general histology and immunohistochemically stained using the avidin–biotin–peroxidase complex (ABC) method. Briefly, the sections were treated by microwave in target retrieval solution (1:10, S1699; Dakocytomatin, CA, USA) for 15 min and then immersed in methanol containing 0.3% H₂O₂ for 10 min at room temperature (RT) to block endogenous peroxidase activity. Afterward, the sections were incubated with normal goat serum (1:50, S-1000, Vector Laboratories, CA, USA) for 30 min at RT to reduce nonspecific staining and incubated overnight with mouse monoclonal anti-bovine tumor necrosis factor- α primary antibody (1:400, 2.5 μ g/mL, MCA2334, Bio-Rad) at 4°C in a humidified chamber. After being incubated with the primary antibody, the sections were incubated with biotinylated goat anti-mouse IgG (1:200, 7.5 μ g/mL, BA-9200, Vector Laboratories) for 30 min, and then ABC reagent was applied for 30 min (1:2, PK-6100, Vectastain Elite ABC kit, Vector Laboratories). The binding sites were visualized with Tris–HCl buffer (pH 7.4) containing 0.02% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.006% H₂O₂. After incubation, the sections were washed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and the nuclei were counterstained with hematoxylin. Slides were dehydrated in a graded series of ethanol, cleared in xylene, cover slipped and observed with a conventional light microscope (Nikon, Microphot-FX), and images were obtained (Nikon, Digital Sight DS-SM). The negative control sections omitted the primary antibody. HE-stained sections were observed using an all-in-one fluorescence microscope (Keyence, BZ-X800).

Immunofluorescence analysis

Endometrial sections were fixed and blocked as described earlier, followed by incubation overnight at 4°C with mouse monoclonal anti-bovine tumor necrosis factor- α primary antibody (1:400, 2.5 μ g/mL, MCA2334, Bio-Rad). The sections were then stained with DAPI (1:50, 340-07971, Dojindo Laboratories, Japan) for 30 min followed by labeling with the secondary antibody (1:200, 5 μ g/mL goat anti-mouse

IgG labeled with Alexa Fluor, Invitrogen, Thermo Fisher Scientific) for 30 min. Sections were washed, and coverslips were mounted using VECTASHIELD mounting medium (H-1000; Vector Laboratories). The fluorescence signal was then captured using an all-in-one fluorescence microscope (Keyence, BZ-X800) using the BZ-X GFP (OP-87763) and BZ-X DAPI (OP-87762) filters set for green and blue wavelengths respectively. Exposure time was kept constant for the primary antibody and its negative antibody control.

Isolation of PMNs from blood

PMNs were isolated from whole bovine blood from three animals according to a protocol described previously (Marey et al. 2014). Heparinized blood was collected from a Holstein cow and mixed with an equal volume of PBS, then gently layered over Ficoll-Paque solution (Lymphoprep, Axis-Shield, Oslo, Norway) and centrifuged at 1000g for 30 min at 10°C. Then the PMN layer was mixed with ammonium chloride lysis buffer (155 mM NH₄Cl, 3.4 mM KHCO₃ and 96.7 µM EDTA) for 10 s and centrifuged at 500g for 10 min at 10°C to purify PMNs from red blood cells. Finally, the isolated PMNs were washed twice with PBS.

Scanning electron microscopy

The preovulatory phase endometrial explants and 10⁶ cells/mL washed fresh sperm were incubated for 30 min and prepared for SEM. Explants without sperm served as controls. To evaluate the effects of TLR1/2 antagonist on sperm entering uterine glands, the preovulatory phase explants were pre-incubated with 1 µM TLR1/2 antagonist in a 38.5°C incubator with 5% CO₂ in humidified air for 30 min. Then the explants were incubated with 10⁶ cells/mL sperm for 30 min. Explants without TLR1/2 antagonist treatment served as controls. Both of these experiments were repeated two times using endometrial explants from two different uteri. Explants were gently washed in TALP and fixed in PB 10% formalin.

Isolated PMNs were placed on a cover glass coated with 0.1% neoprene in toluene, dried at RT and fixed in 2.5% glutaraldehyde in 0.1 M PB. After fixation, the samples were washed in PBS, post-fixed in 1% osmium tetroxide in PBS 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 High-Technologies, Tokyo, Japan). The dried samples were mounted on stubs and sputter coated with Pt using an ion sputter (E-1045; Hitachi High-Technologies, Tokyo, Japan). The samples were observed using a scanning electron microscope (S3400N; Hitachi High-Technologies) at an accelerating voltage of 5 kV.

Isolation of RNA and analysis of mRNA by quantitative real-time PCR

Endometrial explants were homogenized in TRIZOL reagent using an ultrasonic processor (Sonics and Materials, CT, USA) under ice-cold conditions, and further total RNA extraction

was performed using TRIZOL reagent according to a protocol described previously (Chomczynski & Sacchi 1987). The concentration and purity of extracted RNA were determined using NanoDrop Spectrophotometer (2000c, Thermo Scientific) by the absorbance at 260 nm and the 260/280 nm ratios respectively. After measurements, the RNA was stored at –80°C in the RNA storage solution (Ambion, Austin, TX, USA) until cDNA synthesis.

The cDNA was synthesized following a protocol described previously (Yousef et al. 2016) with minor modifications. First, a DNase treatment step was performed using RQ1 RNase-free DNase kit (Promega) to remove residual genomic DNA and other contaminants through which 1 µg extracted RNA was incubated for 30 min at 37°C in a thermal cycler (Eppendorf, Hamburg, Germany) with a first mixture consisting of 1 µl RQ1 RNase-free DNase, 10x reaction buffer, 2 µl RQ1 RNase-free DNase and nuclease-free water (Invitrogen) to a final volume of 10 µl, followed by the addition of 1 µl RQ1 DNase Stop solution for 10 min at 65°C to terminate the reaction. After that, the first-strand cDNA was synthesized according to the commercial protocol described in the SuperScript® II Reverse Transcriptase kit (Invitrogen). Simply, the DNase-treated RNA was incubated at 65°C for 5 min with a second mixture consisting of 1.5 µl of 3 µg/µl random primer, 1.5 µl 10 mM PCR nucleotide mix (dNTP) (Roche Diagnostics) and nuclease-free water to a final volume of 18 µl. In this sequence, a third mixture consisting of 6 µl of 5x first-strand buffer, 3 µl of 0.1 M dithiothreitol and 1.5 µl of 40 units/µl Ribonuclease Inhibitor Recombinant (Toyobo, Osaka, Japan) was added per each tube and then incubated at 42°C for 2 min, followed by the addition of 0.2 µl of 200 units/µl SuperScript™ II Reverse Transcriptase, and the thermal cycler was programmed at 25°C for 10 min, 42°C for 50 min and then 70°C for 15 min. The synthesized cDNA was stored at –30°C.

Quantitative real-time PCR was carried out by a MiniOpticon (Bio-Rad Laboratories) using SYBR Green PCR Master Mix (Bio-Rad Laboratories). Simply, a total 10 µl reaction mix consisting of 2 µl/sample synthesized cDNA, 5 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.2 µl of the targeted primer pairs (Table 1) and 2.8 µl nuclease-free water (Invitrogen) was prepared. The amplification program was run with an initial activation step (15 min at 95°C), followed by 40 cycles of PCR (15 s denaturation at 95°C, 15 s annealing at 55–58°C and 30 s extension at 72°C). A negative control, reactions containing nuclease-free water or non-reverse transcribed RNA, was incubated in each run. The primer pairs were designed by Primer Express® Software v3.0.1 (Thermo Scientific). The melting curve was evaluated at the end of the run to observe the specificity of the amplification. The calculated cycle threshold (Ct) values were normalized using B-actin as an internal control. Fold changes in relative mRNA expression were determined using the Delta-Delta comparative threshold method (Livak & Schmittgen 2001). The housekeeping gene, *β-actin*, was used as an internal standard for the normalization of Ct values because its mRNA expression was stable in all experiments; no significant variations were observed in its mRNA expression across the different treatments.

Table 1 List of the primers used in real-time PCR.

Gene	Sequence of nucleotide (5'→3')		Accession no.	Fragment size (bp)
	Forward	Reverse		
<i>β-Actin</i>	TCACCACTGGGACGACATG	CGTTGTAGAAGGTGTGGTGCC	NM_173979.3	51
<i>IL8</i>	CCAATGGAAACGAGGTCTGC	CCTTCTGCACCCACTTTTCCT	NM_173925.2	51
<i>TNFA</i>	CAAAAGCATGATCCGGGATG	TTCTCGGAGAGCACCTCCTC	NM_173966.3	51
<i>IL1B</i>	AATCGAAGAAAGGCCCGTCT	ATATCCTGGCCACCTCGAAA	NM_174093.1	51
<i>PGES</i>	AAAATGTACGTGGTGGCCGT	CTTCTCCGCAGCCTCACTT	NM_174443.2	51
<i>TLR2</i>	CATGGGTCTGGGCTGTCATC	CCTGGTCAGAGGCTCCTTCC	NM_174197.2	51

Statistical analysis

GraphPad Prism 5 software (GraphPad Software) was used for statistical analysis. Student's *t*-test was applied to compare the mean differences between two groups, and one-way ANOVA followed by Tukey's tests was used to compare the mean differences for more than two groups. Each experiment was repeated five times using explants from different cows. Each group has three replicates, and all the individual values were pooled and used for statistical analysis. All values are presented as mean ± S.E.M. Data were considered to be statistically significant at *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Uterine glands are intact in preovulatory bovine endometrial explants

In the fresh explants of intercaruncular endometrium, a thick, clear layer of mucus could be seen on the surface epithelium. The mucus was viscoelastic and could not be removed by washing the explants in medium.

The histological sections of explants stained with HE showed normal tissue architecture (Fig. 1A). The surface epithelial layer was intact, and uterine glands were present throughout the endometrium. The tubular uterine glands maintained the normal structure

(Fig. 1A and B). SEM images of whole explants clearly show the funnel-shaped entrance into a uterine gland (Fig. 1C).

Sperm glided over the surface epithelium of explants and entered uterine glands

As early as 5 min after the addition of sperm to preovulatory explants, fluorescence video microscopy of sperm with JC-1-stained midpieces revealed that the sperm glided over the surface epithelium until they encountered a uterine gland. There, they stopped within the funnel-shaped opening of the gland, while their flagella continuing beating (Video 1). Next, we used autofluorescence microscopy to see motile sperm within glands after 30 min incubation of sperm with explants (Fig. 2A and Video 2). In this video, the glands are viewed at a slight angle, such that sperm within the glands can be seen clearly. Both videos showed sperm in all visible glands.

Video 1.

JC-1 stained mitochondria in sperm midpieces illuminate sperm that glide over the endometrial surface 5 min after the addition of the sperm to a preovulatory

Figure 1 (A) A section of an endometrial explant stained with haematoxylin and eosin (HE) shows the uterine glands (UGs) in the endometrium. SE, surface epithelium, GO, gland opening, BV, blood vessels. Bar = 200 µm. (B) A fluorescence image shows the presence of UGs in an unstained endometrial explant, revealing autofluorescence under a BZ-X GFP (OP-87763) filter set. Bar = 100 µm. (C) SEM micrograph shows the funnel-shaped UG opening to the uterine lumen. Bar = 5 µm. (D) Sperm appear only within or on the rims of UGs and not attached to the surface epithelium. Sperm tails are visible in the UGs. Bar = 10 µm.

Figure 2 (A) JC1-stained midpiece mitochondria of sperm within the preovulatory phase uterine glands after 30 min incubation with explants of preovulatory endometrium. Bars = 50 μ m. (B) Time-dependent effect of sperm on the relative mRNA expression of proinflammatory cytokines, *PGES* and *TLR2* in the preovulatory bovine endometrial explants. Explants had been incubated with 10^6 sperm/mL for 0, 0.5 and 2 h. Data are presented as mean $\pm$ S.E.M. of five independent experiments. Three uterine explants from one cow were used in each experiment. ** $P < 0.01$, *** $P < 0.001$ denote significant difference (control versus sperm group at the same time point). Different letters denote a significant difference ($P < 0.05$) for the sperm groups compared at different time points.

endometrial explant. Sperm can be seen stopping at the openings of two glands that are side by side at the left side and center of the video. This was due to the binding of the heads to the epithelial surface of the funnel-shaped entrance of the glands (Fig. 1C). The fluorescence video was taken using BZ-X TexasRed (OP-87765) filter set and then converted to gray scale using ImageJ (version 1.51j8). The video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-19-0414/video-1>) from the online version of the article is available at <https://doi.org/10.1530/REP-19-0414>.

Video 2.

Thirty minutes after the addition of sperm to an explant, the beating of the tails of sperm held within two glands can be seen. The fluorescence video was taken using BZ-X GFP (OP-87763) filter set to see autofluorescence and then converted to grayscale using ImageJ (version 1.51j8). This is why sperm heads and the epithelial lining of the glands can be seen in this video. The video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-19-0414/video-2>) from the online version of the article is available at <https://doi.org/10.1530/REP-19-0414>.

SEM revealed greater details of the sperm and glands. The heads of sperm within glands were in contact with the glandular epithelium (Fig. 3A) and/or other sperm (Fig. 3B) and the tails protruded from the glandular openings into the uterine lumen (Fig. 1D). Sperm appeared to associate with microvilli in the glandular

cells (Fig. 3A). Occasionally, a ciliated epithelial cell could be seen near or in glands, but sperm were not found associated with the cilia.

PMNs appeared in the glandular lumen among the sperm

After sperm entered the glands, additional cells were seen in the glands among the clusters of sperm (Fig. 3A). The cells (Fig. 3B) were identified as PMNs, because their size and morphology were identical to PMNs isolated from bovine blood (Fig. 3C). Some PMNs appeared to bind to sperm (Fig. 3B). Neither PMNs nor other nonepithelial cells were seen in the glands in the absence of sperm (Fig. 1C).

Sperm upregulated mRNA expression in the preovulatory explants

Incubation of sperm with preovulatory endometrial explants for only 30 min resulted in an increase in interleukin 8 (*IL8*), which is a strong chemokine for PMNs. *IL8* expression increased throughout the 2 h co-incubation ($P < 0.05$) (Fig. 2B). Co-incubation for 2 h also resulted in the significant upregulation of the proinflammatory cytokines tumor necrosis factoralpha (*TNFA*) and interleukin 1-beta (*IL1B*), as well as increases in prostaglandin E synthase (*PGES*) and *TLR2* mRNA expression.

At 30 min uterine explant incubation with heat-inactivated sperm, no sperm were seen within uterine

Figure 3 Scanning electron microscopy (SEM). The preovulatory phase endometrial explants were incubated with 10^6 sperm/mL for 30 min and prepared for SEM. (A) PMNs and sperm within a UG. Note that sperm are attached to the glandular epithelial cells (arrowhead). Bar = 5 μ m. (B) PMNs binding to sperm at the opening of a UG. Note that a single ciliated surface epithelial cell can be seen (arrowhead); the remainder of the surface epithelial cells show microvilli on their apical surfaces. Bar = 5 μ m. (C) Bovine PMNs collected from blood. Bar = 5 μ m. (D) Mean $\pm$ S.E.M. number of PMNs per UG in endometrial explants incubated for 30 min with and without sperm. Data are presented as mean $\pm$ S.E.M. of two independent experiments. Ten glands were counted per each group. *** P < 0.001 denote a significant difference.

Figure 4 Relative mRNA expression of *IL8* and *TNFA* in preovulatory phase bovine endometrial explants incubated with 10^6 cells/mL of fresh sperm, fresh heat-inactivated sperm and without (control) sperm for 2 h. Data are presented as mean $\pm$ S.E.M. of five independent experiments. Three endometrial explants from individual cow were used in each experiment. ** P < 0.01, *** P < 0.001 denote significant difference.

glands. Moreover, heat-inactivated sperm did not affect the mRNA expression of *IL8* or *TNFA* (Fig. 4).

When live, motile sperm were incubated with luteal phase endometrial explants, the sperm did not appear to enter the glands. Furthermore, the sperm did not alter the mRNA expression of *IL8*, *TNFA* or *IL1B* in the luteal phase explants (Fig. 5). It should be noted that sperm were seen attaching to the extracellular matrix of the connective tissue on the basal surface of the explants. *In vivo*, sperm would not normally encounter this connective tissue, which would not be exposed at the surface of the endometrium. We eliminated the possibility that this interaction of sperm with connective tissue was an initiator of the changes in mRNA expression we observed, because mRNA expression changed only with the exposure of preovulatory explants to sperm, in which sperm entered the glands, and not when luteal explants were exposed to sperm, in which sperm did not enter glands.

Sperm upregulated *TNFA* protein expression in uterine glands

TNFA protein expression was detected using both immunofluorescence and immunohistochemical staining. Both staining methods showed that, when sperm were incubated with uterine explants for 4 h, sperm upregulated *TNFA* expression in uterine glands (Fig. 6A and B).

TLR2 antagonist reduced sperm numbers in glands and inhibited gene expression

SEM images (Fig. 7A) and fluorescence video microscopy revealed that the addition of TLR1/2 antagonist prior to adding sperm subsequently reduced the sperm numbers

Figure 5 Relative mRNA expression of proinflammatory cytokines in luteal phase bovine endometrial explants incubated with 10^6 sperm/mL and without (control) sperm for 2 h. Data are presented as mean $\pm$ S.E.M. of five independent experiments. Three endometrial explants from individual cow were used in each experiment.

in the glands ($P < 0.001$) (Fig. 7B) and inhibited the increase of *TNFA* gene expression seen in the presence of sperm (Fig. 8). The treatment of preovulatory explants with TLR1/2 antagonist alone for 2 h did not change the gene expression of *IL8*, *TNFA*, or *IL1B* in the explants (Fig. 9).

Discussion

Our explant culture model provided the first direct visual evidence that sperm glide over the endometrial surface and enter uterine glands. Both the videos of fluorescently labeled sperm and the SEM images demonstrated that sperm did not bind to the surface epithelium of the endometrial explants, but they entered and were held in the glands. The mucous layer on the surface epithelium may have enabled the sperm to glide smoothly over the epithelial cells until sperm came into contact with the openings of uterine glands. Further, due to the established hydrodynamics of sperm near-surface swimming, bull sperm would be expected to remain swimming along the surface epithelium (i.e. not swimming away from the surface) and to enter the glands when they were encountered (Denissenko *et al.* 2012,

Tung *et al.* 2015). The SEM images indicate that sperm within the glands attach to microvilli of the glandular epithelium, which may be part of the mechanism that held sperm within the glands.

Sperm retention in the uterine glands has been reported in many mammalian species, such as guinea pig, stoat, hedgehog, mole (Austin 1960), dog (Doak *et al.* 1967), sow (Lovell & Getty 1968), rabbit (Koyama *et al.* 1986) and bat (Racey *et al.* 1987). However, to our knowledge, there are no reports of studies that link sperm retention in glands with uterine immune responses. Therefore, ours is the first report that sperm in uterine glands stimulate innate immune responses.

Our SEM images indicated that sperm bound by their heads to the glandular epithelium, as well as to each other. Furthermore, sperm entrapment in the glands seemed to be strong, because sperm were retained in glands after washing the explants. This observation is supported by a previous study, where many more sperm were detected in the canine uterus by histology and SEM than by uterine flushing, indicating that sperm were held tightly in uterine glands (Rijsselaere *et al.* 2004). Glandular retention of sperm has been shown in the histological sections of bovine uterine glands 24 h after

Figure 6 TNFA expression in endometrial explants, particularly in uterine glands after the incubation of bovine uterine explants for 4 h with and without sperm using (A) immunofluorescence labeling with Alexa Fluor-conjugated anti-TNFA antibody, and (B) immunostaining with peroxidase-conjugated anti-TNFA antibody, visualized using DAB (brown). The primary antibody was omitted in the negative control sections. Nuclei are counterstained with hematoxylin. Bars (A) = 50 μ m, (B) = 20 μ m.

Figure 7 TLR2 antagonist reduced sperm numbers in uterine glands. (A) SEM images of bovine endometrial explants that had been incubated with sperm $\pm$ TLR1/2 antagonist. The preovulatory phase endometrial explants were pre-incubated with 1 μ M TLR1/2 antagonist for 30 min, followed by the addition of sperm and incubation for an additional 30 min. Bars = 10 μ m. (B) Number of sperm/gland in explants incubated with sperm $\pm$ TLR1/2 antagonist. Data are presented as mean $\pm$ S.E.M. of five independent experiments. For each treatment, sperm were counted in three uterine glands. *** P < 0.001 denote a significant difference.

AI (Koyama *et al.* 1986), indicating that sperm retention lasts for several hours *in vivo*.

The percentage of sperm retained in the glands was low when compared to total sperm added. We estimated roughly 0.5% of the sperm added to the explant culture wells were trapped in the glands. This calculation was based on the numbers of sperm added to the wells, the mean number of glands per explant and the mean numbers of sperm counted within representative glands. Nevertheless, because the physical dimensions of the uterine lumen and endometrial surface are different from the physical dimensions of our explant model with 8 mm endometrial tissue disks, it is not possible to extrapolate our data to the percentage of sperm that enter glands *in vivo*.

Our data indicate that sperm interaction with the glandular epithelium triggers local uterine innate immune responses via the upregulation of proinflammatory cytokines. Sperm upregulated mRNA expression of proinflammatory cytokines *IL8* (at 0.5 h) and *TNFA* and *IL1B* (at 2 h) in whole explants. Previously, our group reported that frozen-thawed motile sperm upregulated similar genes in BUECs, in which the uterine epithelium was somewhat dedifferentiated and epithelium could not be distinguished from the glandular epithelium (Elweza *et al.* 2018). Further, in the present study, we detected the synthesis of TNFA protein, a strong proinflammatory marker, in the glandular epithelium. Our immunohistochemistry results indicated that TNFA protein was expressed mainly in the glandular

Figure 8 Relative mRNA expression of proinflammatory cytokines in preovulatory phase explants incubated with sperm, with sperm + TLR1/2 antagonist and without sperm or antagonist for 2 h. Data are presented as mean $\pm$ S.E.M. of five independent experiments. Three explants from individual cows were used in each experiment. * P < 0.05, ** P < 0.01, *** P < 0.001 denote significant difference.

epithelium. We cannot completely exclude the possibility that luminal epithelium is also involved in the inflammatory response, as mRNA expression was measured in the whole explant. Nevertheless, it should be noted that most of the sperm we observed *in vitro* glided over the mucus-coated endometrial surface rather than adhering to endometrial cells. These observations suggest that sperm entering glands is one of the main triggers of the uterine immune response, if not the only one. Further investigations are necessary to clarify the

Figure 9 Relative mRNA expression of proinflammatory cytokines in preovulatory phase explants incubated with $\pm$ TLR1/2 antagonist for 2 h. Data are presented as mean $\pm$ S.E.M. of five independent experiments. Three explants from individual cows were used in each experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denote significant difference.

role of luminal epithelium in activating the inflammatory cascade induced by sperm.

Our SEM images showed that PMNs were present in the glands. PMNs are known to rapidly enter the uterine lumen after the insemination of cows (Alghamdi *et al.* 2009), as well as of females of several other species of mammals (Mattner 1968, Katila 1995, Rozeboom *et al.* 1998, England *et al.* 2013). Our experiments indicate that, in the case of cattle, the initial route of PMNs into the uterine cavity is from glands. The source of the PMNs would be the endometrial stroma, which was present in our explants. It has been reported that, during pro-estrus and estrus in cattle, PMNs assemble in large numbers in the endometrial stroma (Mattner 1968). After PMNs enter the glands, attachment of sperm to PMNs could upregulate the innate immune response by triggering the PMNs to produce immune-regulatory cytokines, which can, in turn, increase additional PMN migration, including additional migration through the surface epithelium.

The PMNs in the bovine uterine glands could phagocytize sperm. PMNs have been observed phagocytizing uterine sperm in other species such as in mice, rats (Austin 1957), goats (Mattner 1968), pigs (Pursel *et al.* 1978), and rabbits (Bedford 1965). PMN phagocytosis of sperm within uterine glands was reported in little brown bats, guinea pigs, horseshoe bats, rabbits and pigs (Austin 1960, Koyama *et al.* 1986, Racey *et al.* 1987). In addition to PMNs, there is some evidence that macrophages phagocytize sperm within uterine glands in rabbits and cows (Koyama *et al.* 1986). Altogether, phagocytosis within the glands and the uterine lumen is thought to clear sperm from the uterus in preparation for the arrival and implantation of embryos (Katila 2012).

Our observations revealed that sperm entered and remained in the preovulatory phase uterine glands but not luteal phase glands. It seemed that fewer sperm were gliding over the surface epithelium of luteal endometrium; however, the sperm that were seen did not enter into the glands they encountered. In the luteal phase, under progesterone dominance, uterine glands are known to become enlarged, tortuous and full of secretion (Ohtani *et al.* 1993, Wahab *et al.* 1999, Dhaliwal *et al.* 2002, Wang *et al.* 2007). Although the physical attraction of sperm for surfaces would tend to direct them into glands, as discussed earlier, the physical and/or chemical nature of the luteal phase secretions in the glands may have prevented them from doing so.

Previously we reported that the addition of CU-CPT22, a TLR1/2 antagonist (Cheng *et al.* 2012), into BUEC monolayer cultures suppressed the proinflammatory response of BUECs to sperm and the phosphorylation of JNK as a downstream target of the TLR2 signaling pathway (Ezz *et al.* 2019). To check the involvement of TLR2 in the explant response to sperm, we tested the effects of the antagonist on explant response to sperm. Pre-incubation of uterine explants with antagonist reduced sperm retention in the uterine glands and prevented the increased expression of the inflammatory cytokine TNFA. Further investigations are necessary to elucidate the detailed mechanism of the activation of TLR2 signaling pathway by sperm.

It can be concluded that our bovine explant culture model can be used to investigate the initial sperm–uterine interactions and immune responses. Here, the culture model provided evidence that sperm entry into uterine glands triggers the innate immune response. A next step would be to determine whether this initial response in the glands eventually leads to a massive immigration of PMNs into the stroma and then through the surface epithelium to the uterine cavity to ensure that the cavity is thoroughly cleared and prepare for the arrival of the embryo.

Declaration of interest

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

Funding

The present research work was funded by a Grant-in-Aid for Scientific Research (16H05013, 17F17407 and 18K19259) of Japan Society for the Promotion of Science (JSPS), Kieikai Research Foundation (2018C015 and 2019C055) and in part by Livestock Promotional Funds of Japan Racing Association (JRD).

Author contribution statement

I A, S S S, M S⁶ and A M conceived and designed the experiments; I A, S S S, V A M, M S¹, M A E and M A M performed the experiments; I A, S S S, V A M, M A E and M A M analyzed the data; M S¹, K T and A M provided reagents/

materials/analysis tools; and I A, S S S, M S⁶ and A M wrote the manuscript. All authors reviewed the manuscript.

Acknowledgements

We thank Anni Nurliani, Veterinary Anatomy, Obihiro University, Japan, for her help in immunocytochemistry and scanning electron microscopy.

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Received 31 August 2019

First decision 9 October 2019

Revised manuscript received 10 November 2019

Accepted 2 December 2019