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Abstract of Dissertation

Applicant

Doctoral Program in Animal and Food Hygiene

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Title : **Dynamics of sperm-uterine interaction that initiates a maternal pro-inflammatory response in the cow**

（ウシ母体の炎症反応を起動する精子と子宮の相互作用におけるダイナミクス）

Abstract

In mammals, millions of sperm enter the uterus after mating. From there, thousands pass into the oviduct in most species. In the course of migration through the female tract, sperm interact with the walls of the tract, as well as with immunological cells that enter the lumen of the tract. Sperm interaction with the uterus and subsequent immune responses by the endometrium to remove excess, defective and dead sperm are important to prepare the endometrium for embryo implantation. Little is known about whether and how the interaction of sperm with endometrium causes inflammatory responses in bovine. Our group recently showed that sperm binding to bovine uterine epithelial cells induces an acute inflammatory response and this involves Toll-like receptors (TLRs) signaling pathway *via* TLR2. However, physiological and immunological functions of the uterus depend on interactions of various tissues, presence of mucus, extracellular matrix, as well as the differentiation state of the cells. Thus, an *ex vivo* explant model was developed to investigate the sperm-uterine interaction and immune responses *in vivo*.

In chapter 1, a bovine endometrial explant culture model was developed to study the sperm-uterine interactions and immune responses. Using healthy reproductive tracts of cows, the ipsilateral horns relative to the ovary containing the mature follicle of the pre-ovulatory phase were

isolated. Using an 8 mm biopsy punch, disks of endometrial tissue (2 mm thick) were dissected from the intercaruncular endometrial regions. Then explants were incubated with 10^6/ml washed fresh sperm in Tyrode Albumin Lactate Pyruvate (TALP) medium in a 38.5°C incubator with 5% CO_2 in humidified air. Mitochondrial stain JC1 labeled sperm were used in fluorescence microscopy. Scanning electron microscopy (SEM) images were taken after the co-culture of explants with sperm. Sections of bovine pre-ovulatory endometrial explants stained with hematoxylin and eosin showed normal tissue architecture with intact surface epithelial layer and simple tubular uterine glands that present throughout the extracted endometrium. SEM images of whole explants clearly show the funnel-shaped entrance into a uterine gland. Fluorescence video microscopy revealed that the sperm glided over the surface epithelium until they encountered a uterine gland. Further, SEM images revealed that the heads of sperm within glands were in contact with the glandular epithelium and/or other sperm, and the tails protruded from the glandular openings into the uterine lumen. The developed *ex vivo* explant culture model provides greater details of sperm behavior and dynamics with endometrial explants. Particularly, it shed evidence that uterine glands serve as a site where sperm interact with glandular epithelium. Therefore, the developed bovine *ex vivo* explant culture model can be successfully used to investigate the dynamics of sperm-uterine interactions.

In chapter 2, a detailed study on sperm-uterine interaction on immune responses was carried out using the developed bovine endometrial explant culture model. There are no reports of studies that link sperm retention in glands with uterine immune responses. Therefore, this is the first report that sperm in uterine glands stimulate innate immune responses. 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 Polymorphonuclear neutrophils (PMNs). *IL8* expression to increase throughout the 2 h co-incubation. Co-incubation for 2 h also resulted in significant upregulation of the pro-inflammatory cytokines Tumor Necrosis Factor Alpha (*TNFA*) and Interleukin 1-beta (*IL1B*), as well as increases in Prostaglandin E Synthase (*PGES*) and *TLR2* mRNA expression. Further, 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. SEM observations show that endogenous PMNs appeared in uterine glands along with the sperm after sperm entered the glands; they may initiate sperm clearance. To investigate the effect of heat-inactivated sperm, the pre-ovulatory phase explants were incubated with 10^6 heat-inactivated sperm/mL. At 30 min of uterine explant incubation

with heat-inactivated sperm, no sperm were seen within uterine glands. Moreover, heat-inactivated sperm did not affect the mRNA expression of *IL8* or *TNFA*. 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 mRNA expression of *IL8*, *TNFA*, or *IL1B* in the luteal phase explants. As a summary, the culture model provided evidence that sperm entry into uterine glands triggers the innate immune response. Therefore, the developed bovine explant culture model can be used to investigate the initial sperm-uterine interactions and immune responses. Further, the observations suggest that sperm entering glands is one of the main triggers of the uterine immune response, if not the only one. Additional investigations are necessary to clarify the role of other tissues in endometrium such as luminal epithelium in activating the inflammatory cascade induced by sperm.

In chapter 3, to study the mechanism of sperm uterine interactions, the developed endometrial explant culture model was used. Among other TLRs, TLR2 has the ability to mediate inflammation at the interaction of sperm with cumulus-oocyte complexes during fertilization. Further, recently our group reported that sperm binding to monolayer cultures of bovine uterine epithelial cells induces an acute inflammatory response in the epithelium by TLR signaling pathway via TLR2. Therefore, as an initial step, the involvement of TLR2 in sperm-uterine interactions and immune responses was evaluated. Attachment and pro-inflammatory responses were evaluated after blocking the sperm attachment to glands using the TLR2 antagonist. The pre-ovulatory phase explants were pre-incubated with 1 μM antagonist for 30 min and further explants were incubated with 10^6 sperm/mL for 0.5 h and 2 h for SEM and mRNA evaluations respectively. TLR2 antagonists reduced the sperm numbers in the glands and inhibited the increase of *TNFA* mRNA expression. It suggests that the sperm-uterine inflammatory process is at least partly mediated by TLR2 signaling. Further investigations are necessary to elucidate the detailed mechanism of activation of the TLR2 signaling pathway by sperm.

It can be concluded that the bovine explant culture model can be used to investigate the initial sperm-uterine interactions and immune responses. The culture model provided evidence that uterine glands serve as a site where sperm interact with glandular epithelium to trigger the uterine inflammatory cascade which begins in the glands to rapidly clear sperm from the uterus and to prepare the endometrium for the arrival of the embryo. Further investigations are necessary to find the possible mechanism/chemoattractants from the uterine glands which attract the sperm and also to explore the possible sperm- TLR2 ligands involved in the interactions and immune responses.