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

2019

MOHAMMADU AKUTHAR Muhammed Ihshan

Doctoral Program in Animal and Food Hygiene

Graduate School of Animal Husbandry

Obihiro University of Agriculture and Veterinary Medicine

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

令和元年

（2019）

帯広畜産大学大学院畜産学研究科

博士後期課程　畜産衛生学専攻

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General Introduction

With the increasing population all over the world, it is important to improve food production to supply human needs. Among various industries, dairy and beef industries play major roles in fulfilling this need. Nowadays the main aim of these industries is to improve the fertility of cows. They face many obstacles, such as artificial insemination (AI) failures and poor estrus synchronization while they working towards their goals. A deeper understanding of reproductive physiology is vital to overcome such problems. Among various factors, sperm-egg interaction is a vital phenomenon to improve fertility. In bovine, after natural mating or AI, plenty of sperm released into the uterus in which only several passes into the oviduct to reach their goal; female gamete. In the course of sperm journey from the uterus to fertilizing site, they need to interact with many portions of the female tract. We know surprisingly little about how sperm interact with the female reproductive tract (FRT) while migrating to the eggs. Some of the problems faced by reproductive biologists could be overcome if more were known about sperm interactions with the FRT. This information would be useful to optimize the processes of semen extension, freezing, and AI.

1. Sperm

The process spermatogenesis occurs in the testis for the production of sperm. The sperm of different species vary widely in size and shape; For example, bull and human sperm have paddle-shaped heads and rodent sperm have hook-shaped heads. Sperm mainly consists of sperm head, sperm midpiece, and sperm tail. The heads of bull sperm are about 10 µm long, and 5 µm wide [1]. The sperm head consists of chromatin. The anterior half of the head is covered by the acrosome, which is a membrane-enclosed sac of enzymes important in the penetration of the egg by the sperm. The bovine, even though having larger sperm nuclear areas, displayed clearly smaller acrosomes than those of the ovine and porcine [1]. The whole sperm including the acrosome is enclosed by a plasma membrane. The sperm midpiece condensed with mitochondria which provide the energy for the sperm. Sperm tail particularly helps in progressive motility.

2. Female reproductive tract of a cow

The FRT of a cow consists of several major sections such as external genitalia-vulva, vagina, cervix, uterus, oviducts, and ovaries. In bovine, FRT is positioned below the rectum. The FRT

facilitates many major functions, such as production of female gametes, transportation of male and female gametes, fertilization, development of the embryo into fetus, and delivery of the neonatal. Further, the sections of the FRT are subject to being colonized by pathogens and provide a pathway into the body cavity. Therefore, the FRT has many adaptations with an active immune system to protect itself from invasion of pathogens and microbial encounters.

2.1. Uterus

The uterus is a muscular hollow structure that takes part in many reproductive functions such as, providing space for embryo and fetus to grow and mature, nurturing the pre-implanted embryo and providing the ideal situation for its development, serving by uterine contractility to expulse both the neonatal and placenta. Further, it provides the pathway for sperm to move towards the oviduct. The uterus consists of cervix, body, and two horns. Uterus attaches cranially to the oviducts, and caudally to the vagina. The uterus consists of perimetrium, myometrium, and endometrium. Perimetrium is positioned in the outside of the uterus surface. Myometrium is made of smooth muscle fibers. The endometrium is the mucosal organ which varies frequently by hormonal changes from stage of the estrous cycle and during pregnancy. In some areas of the endometrium there are about seventy to hundred and twenty caruncles which are the prominent structures, organized in four longitudinal lines [2]. The luminal epithelium is columnar or cubic simple with some ciliated portions. Under the luminal epithelium the stroma is located that is formed by connective tissue containing the uterine glands.

2.2. Uterine glands

Histologically, the endometrium comprises two epithelial cell types, the luminal epithelium (LE) and glandular epithelium (GE) which are two stratified stromal sections including a densely and loosely organized stromal regions, blood vessels, and immune cells. The origin of uterine glands involves differentiation and budding of GE from LE, penetration of uterine stroma by tubes of GE, and extensive coiling and branching of GE [3]. The endometrium in cattle comprises of a large number of elevated aglandular caruncles, dense stromal protuberances covered by a simple LE, and intensively glandular intercaruncular regions [2]. Caruncular regions are the locations of superficial implantation and placentation [4]. Intercaruncular endometrial areas contain large numbers of branched, coiled uterine glands that synthesize and secrete or transport histotroph which consists of a variety of

enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, and other substances [5, 6].

In ovine, extensive hyperplasia and hypertrophy of uterine glands in the stratum compactum occur during each pregnancy [4]. After parturition in the ewe, intercaruncular endometrial LE remains intact, but degeneration and apoptosis of many GE cells have been observed on the day after parturition [7]. Meanwhile, glandular regeneration commenced by day 8 postpartum, and the glands were substantially regenerated by day 15 [7]. Changes in the endometrial morphology along the estrous cycle are more evident in the GE and stroma than in the LE. At the beginning of the follicular (proliferative) stage endometrium is thin, stroma is loose, and low-simple columnar glands are rare and straight, lumen is narrow. Later on, vascularization increases, stromal, luminal, and glandular cells display mitosis; number of both stromal and glandular cells increases and edema is present. At the end of this stage, stroma becomes dense, glands are bigger in number, elongated, and sinuous, epithelium looks higher, pseudostratified and the lumen is empty. In the luteal (secretory) stage endometrium reaches its maximum thickness, blood vessels enlarger remarkably, and glands display secretory capacity. In this stage, glands are abundant, tortuous, and shortened; lumen is wide, full of secretion, and ready to produce histotrophe. GE is columnar simple, although some parts are columnar stratified, and contains a great amount of secretion vacuoles [8, 9].

3. Sperm-female reproductive tract interactions

During the transport of sperm through the FRT, they interact and communicate with various portions of the FRT to ensure that the sperm which has the typical morphology and vigorous motility reach the fertilization site. After sperm enter into the FRT, they; provide the route to the sperm, select the best sperm for fertilization, and store the sperm until the fertilization period. Depending on the species, fertilization occurs immediately, few hours, days or even months after sperm insemination. Therefore, from semen deposition until fertilization sperm must survive in the FRT.

3.1. Sperm interaction with the caudal part of the FRT

The site of semen deposition varies depending on the species. In humans, semen pooled in the anterior vagina after coitus and within minutes sperm swim into the cervical canal [10]. In rodents, sperm placed in the vagina, removed through the cervix into the uterus along with seminal plasma

within few minutes [11]. Similarly, in cattle and sheep ejaculate is deposited in the vagina near to the posterior end of the cervix and the time take to enter into uterus expands over more than 24 h [12]. In pigs semen deposits directly into the uterine cavity [13].

In cows, after insemination 60% of the sperm loses through the backflow [12]. In cattle and goats, the cervix has been suggested to serve as a sperm reservoir [14]. Further, it is reported that sperm stay within the crypts and folds of the cervix of cattle [15]. Cervical mucus might serve as a selective mechanism for the abnormal and immotile sperm [16]. But active sperm may use the cervical mucus for the movement towards the uterus.

3.2. Sperm interaction with the uterus

Sperm move into the uterus either directly [13] or after passing through the cervical channel [12]. Sperm-uterine interactions have been studied intensely in porcine when compared to other species. In porcine, it seems that sperm stored and selected in the uterus before they move towards fertilization. The porcine uterus facilitates several millions of sperm for up to 24 h, while few sperm can be found in the oviducts as shortly as 5 to15 min [17]. Porcine sperm were detected bind to epithelial cells [18]. A study using porcine *ex vivo* model revealed that viable and motile sperm bind to uterine epithelial cells [19]. In bovine when inseminated directly into the uterus, within 1 h, 96% of the heat-inactivated sperm had been discharged, while only 26% of the live sperm had been discharged into the vagina [20]. These findings suggest that viable sperm retained within the uterus. The purpose of sperm within uterus is unclear whether for selection or storage. However, a study in humans reveals that sperm binding to uterine epithelial cells increases the sperm motion factors [21]. Another study in porcine suggested that the reservoir in the oviducts is fed restrictively by a larger reservoir in the uterus [22]. However, there is no clear evidence for this storage phenomenon of sperm in the uterus. Further, sperm binding to the uterus may be due to the negative selection mechanism. A study in porcine shown that occurrence of seminal plasma leads to less viable sperm binding to uterine epithelial cells, showing that the binding to uterine wall is harmful to sperm, since seminal plasma commonly considered as a protectant for sperm [19]. Normally, insemination leads to an increase in the migration of PMNs in to the uterus [14, 23]. Previous evidence suggest that interactions of frozen-thawed sperm with bovine uterine epithelial cells starts to produce pro-inflammatory cytokines to alert the immune system [24].

These studies give suggestions that uterus accomplish a selection mechanism to recognize the suitable sperm to reach the oviduct.

3.3. Sperm retention in uterine glands

Sperm retention in the uterine glands has been reported in many animal species, such as guinea pig, stoat, hedgehog, mole [25], dog [26], sow [18], rabbit [27] and bat [28]. But rarely investigated in the cow, where histological observations showed that few sperm were located in the uterine glands 24 h after AI, but sperm in the uterine glands were not observed by SEM [27]. In dogs, sperm were mainly clustered in the uterine glands, most frequently in the neck of these glands following natural mating [26]. Further, in dogs, the majority of the sperm were located in the endometrial glands whereas few sperm were found in the lumina after AI [29, 30, 31]. In sows, the glandular openings were completely filled with sperm after natural mating [27]. In the rabbit, sperm appeared in the uterine glands and few sperm were found in the uterine lumen after natural mating [27]. No differences were observed in the glands with sperm in the different regions of the uterus [30, 31]. A study in the dogs revealed that 24 h after insemination during the ovulatory phase, more than five sperm were seen in the majority of uterine glands. Meanwhile, the uterine glands mostly with one sperm were frequently found when inseminated during the pre-ovulatory phase [30]. However, the percentage of uterine glands with sperm 24 h after natural mating in dogs was higher [26]. The above reports strongly suggest that sperm retention within uterine glands is a common physiological phenomenon that happens *in vivo*.

When the cow was inseminated directly into the uterus, within 1 h, 96% of the heat-inactivated sperm had been discharged into the vagina, while only 26% of the live sperm had been discharged [20]. Active sperm attached to BUECs monolayers and heat-inactivated sperm did not attach to BUECs [24]. Also, another study in ovine claimed that dead sperm did not migrate into the cervical glands [32]. An *in vitro* study in pigs showed that viable sperm retains in *ex vivo* uterine segments [19]. This shed evidence that only the live sperm retains in the uterine glands. Dead sperm which couldn't attach to the glands might be removed by backflow which indeed has been proven to be an effective tool in removing dead sperm [20]. It is also suggested that live sperm penetrate into the glands by their own active force but not by any external forces.

3.4. Sperm interaction with the oviduct

Before entering into the oviduct, sperm have to pass through the narrow passage of utero-tubal junction when compared to the uterus [33]. Further, the lumen of the utero-tubal junction is twisted, and complicated with mucosal folds and grooves. Moreover, a viscous mucus in the lumen of the utero-tubal junction and the neighboring isthmus has been described in bovine [34] and other species. Therefore, sperm have to pass through these obstacles to reach the oviduct.

Once sperm reach the oviduct they have to interact with the chemical and physical factors of the oviduct. Many of the sperm that enter into the oviduct bind to the oviductal epithelium and create a storage reservoir in the oviduct [35]. It was reported using scanning electron microscopy (SEM) that bull sperm bound to cilia on the epithelium of the oviductal isthmus [36]. The fertilizing capacity of sperm maintained by their interaction with oviductal epithelium [37]. Holding sperm in the lower oviduct may also serve to prevent polyspermic fertilization by allowing only a few sperm at a time to reach oocytes in the ampulla [35]. Carbohydrate moieties on the surface of oviductal epithelium play a role in the interactions with the sperm [35]. As the time of ovulation is near, sperm begin to detach from the epithelium, and may then reattach and detach several times before they move out of the storage [38]. Modification of cell surface proteins and hyperactivation of motility of sperm during the process of capacitation helps in detachment [35]. Capacitated bull sperm showed reduced binding to oviductal epithelium [39]. Further, oviductal epithelium plays a role in sperm release by secreting factors that affect sperm [35].

4. Female reproductive tract immunity

The FRT is under threat due to many factors such as sexually transmitted diseases, systemic infections, and chronic inflammatory conditions. In addition to protecting against infections, FRT must adapt to physiological events that include fertilization, implantation, and pregnancy. During pregnancy, in addition to protecting against infections, the mucosal immune system protects the fetus using immunologically distinct unit.

Protection against infectious agents in the FRT is overcome with the help of the immune system which acts through two subunits called innate and adaptive immunity. Adaptive immunity encompasses pathogen-specific defense mechanism. An effective immune response to pathogens

requires that antigen-presenting cells process antigen from the pathogen and present it to T-cells. Following antigen presentation, lymphocyte effector functions, including cytokine production, cytotoxicity, and antibody synthesis are activated. Protection is mediated either through specific antibodies produced by B-cells and/or the destruction of specific pathogens by T-cells [40].

The innate immune system includes rapid, primitive and non-specific responses than the adaptive immune system, such as surface defenses, production of cytokines, complement initiation and phagocytosis [40]. Known as the first line of defense, the innate immune system functions to prevent and regulate the invasion of pathogens. It has developed to identify foreign materials that are not typically present in the host. Further, it is responsible for numerous obstacles of non-specific protection against microbial infection and tissue damage in the FRT. The anatomical obstacles comprise of the vulva, vagina, cervix, the stratified squamous epithelium of the vagina, the columnar epithelium of the endometrium, the basement membrane of ovarian follicles and the zona pellucida of the oocyte [41].

The FRT is provided with an active mucosal immune system which plays two opposite roles, by supporting an allogenic sperm and accepting a semi-allogenic embryo/fetus, and protecting against invading pathogens (Fig. 1) [42].

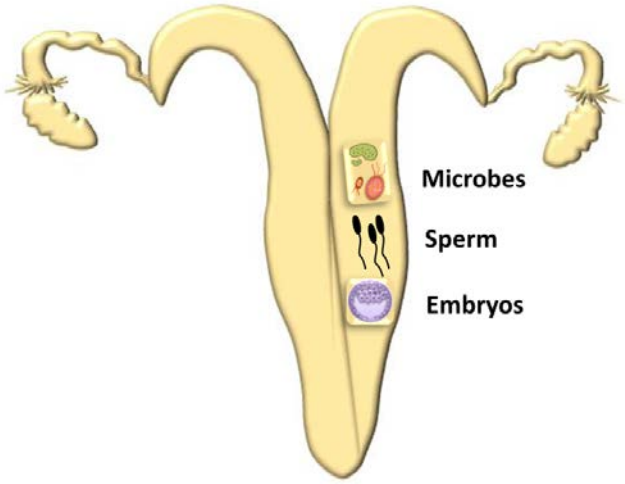

Figure 1. A diagram showing that FRT contained with an acute immune system which plays two roles, by supporting sperm, and embryo, and protecting against pathogens.

4.1. Immunity to sperm in the uterus.

It is well established that the contact of semen components with cervical and uterine tissues elicits a series of immunological responses [43]. Since semen is an external material to FRT, it suspects many of immunological cross-talk in a challenge to exclude the foreign material. Since sperm are

antigenic to females, immunization against sperm can encourage infertility therefore, the rapid removal of sperm is needed to prevent acquired immune responses against sperm [44]. The immune responses activated by semen not only target for rapid elimination of sperm but also affects functional immune tolerance to the paternal antigen for development of preimplantation embryos [45].

4.1.1. PMNs influx after sperm arrival.

Polymorphonuclear neutrophils (PMNs) are the rich cell types of leukocytes in the blood. They are identified by a unique multi-lobulated nucleus and a highly granulated cytoplasm. PMNs are one of the main types of effector cells of the innate immune system. PMNs play an important role in overcoming infection by phagocytosis or by the secretion of antibacterial substances [46].

It is reported that during pro-oestrus and oestrus, PMNs assemble all the way through the uterine endometrium along the basal lamina of the surface epithelium [47] and insemination causes a considerable number of these PMN to proceed through the basal lamina into the surface epithelium and the uterine lumen [48]. PMNs were present in the epithelial stroma of the uterus, immediately beneath the mucosal layer, in goats and cows [14]. The growing population of leukocyte in the uterus after sperm arrival is a common phenomenon in many species. This inflammatory response helps to clear the uterus from excessive sperm and other contaminants associated with mating as well as to prepare an ideal uterine atmosphere for receiving the embryos [49].

In humans [50] and rabbits [51], insemination stimulates the migration of neutrophils and macrophages, into the cervix and vagina. The leukocyte influx after sperm arrival predominantly consisted of PMNs [52]. In goats and cows, a greater number of luminal PMNs were found in the uteri and cervix in mated animals [14]. Sperm, induced PMN influx in horse uterus [23]. In rabbits, PMNs have been reported to migrate into the vaginal lumen post-coitus [53]. Coitus induces a leukocytic infiltration of the uterine cavity, which reaches a peak several hours after mating in mice, approximately 20 h after coitus, the uterine contents are apparently evacuated [54]. In the dog, a significant increase in PMNs in the uterine lumen following insemination of normal bitches, with fresh semen was observed [55]. The presence of sperm in the uterus induces a rapid influx of PMNs, which are detected in the uterus 30 min after insemination in horses and pigs [48, 56]. During another study, the highest number of PMNs in gilts was found 12 h after AI [52]. PMN numbers peak between 4 and 8

h in the mare [56] and 3-6 h in pigs [57]. The numbers remain elevated for 24 h but by 40-48 h only a few PMNs are found in mares and pigs [48, 56]. In the cow, PMNs peak around 8-16 h [58]. But another recent study mentioned, in cows PMNs peak at less than 2 h [59].

Sperm activates a complement cascade in uterine secretions which in turn leads to an influx of PMNs into the uterus [60]. The complementary cascade mediates a series of biological reactions such as vascular permeability, chemotaxis, and opsonization for phagocytosis [49]. Consequently, activated PMNs phagocytize sperm through the formation of DNA-based neutrophil extracellular traps entrapment or by direct cell membrane attachment [59].

4.1.2. PMNs and sperm phagocytosis.

In the cow, phagocytosis of sperm by PMNs was observed in the vagina [58], cervix, oviduct, and uterus [14]. Neutrophils have been observed phagocytizing uterine sperm in other species such as in mice, rats [54], goats [14] and rabbits [61]. Phagocytosis of sperm by PMN was occurring in gilts by 2 h after inseminating [22]. In gilts, the sperm population in the oviducts remained stable for over 24 h meanwhile the population of sperm and PMN in the uterus decreased rapidly [22].

Phagocytosis of sperm by PMNs within uterine glands was rarely observed. But engulfment of sperm by PMN in uterine glands was evident in little brown bats and horseshoe bats [25, 28]. Sperm along with leukocytes in uterine glands also observed in guinea pigs [25]. The phagocytosis of sperm by PMNs within the sow uterine glands was observed [27]. The rapid disappearance of the sperm in the uterine glands was observed in rabbits and sows [27]. The majority of PMNs were observed around and within uterine glands in cows after mating [14]. In another study in dogs, from day two to four after ovulation, a significant decrease in the percentage of glands containing sperm and a marked reduction of the sperm number in uterine horn glands were observed [31].

Also, there is some evidence previously that macrophages phagocytize sperm within uterine glands. It was observed in the cow, low number sperm were phagocytized by migrated macrophages in uterine glands [27]. Sperm engulfed by macrophages were observed also in rabbit uterine glands where the phagocytizing rate increased at 24 h after mating; then the macrophages in the glands decreased after that time [27].

In the cow, the majority of sperm are removed rapidly after semen deposition [12]. The sperm removal process occurs through backflow [20, 62] and phagocytosis mainly by PMNs [52] (Fig. 2). Sperm-neutrophil binding occurs in different ways such as individual neutrophil bound to one or more sperm cells or variable sizes of clustered cells [59]. Presence of neutrophils, which is a marker of inflammation, along with the sperm in the uterine glands after sperm arrival showing an apparent uterine inflammatory action in response to sperm. Neutrophils are capable of inducing apoptosis [63]. So another possible option is, the sperm which retains in the gland could be removed in this way in a shorter time.

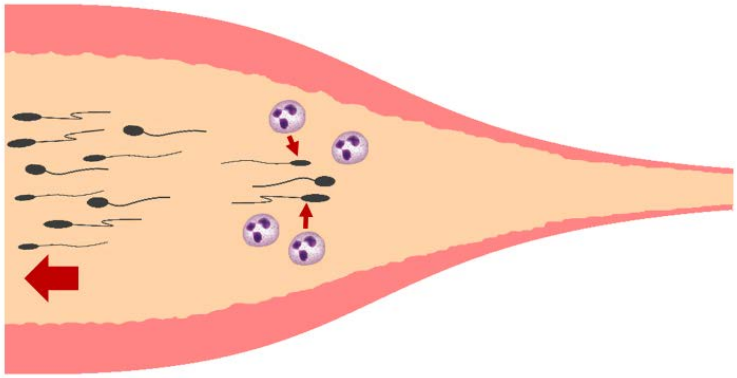

Figure 2. The diagram showing the backflow and phagocytosis of sperm by PMNs in the uterus after semen deposition.

Neutrophils target and remove preferentially aged, damaged, dead, or prematurely capacitated sperm [64, 65, 66]. In the cow, when inseminated with ejaculates had high levels of morphologically abnormal sperm, 12 h after insemination majority of the sperm were determined in the vagina, fewer sperm in uterine horns where no sperm were recovered in the oviduct 12h after insemination [12]. Therefore, it is suggested that phagocytosis of sperm by PMN in uterine glands may affect the removal of defective and excess sperm and allow luminal sperm to reach the oviduct. Further, attachment of sperm to PMN may be important for the regulation of the uterine immune response by triggering the neutrophils to produce immune-regulatory cytokines, which in turn increase additional neutrophil migration into the uterus.

4.1.3. Inflammatory mediators

After the arrival of semen or pathogens into the uterus, several inflammatory mediators accumulate, which modulate the acute immune responses. These mediators include cytokines such as Tumor Necrosis Factor Alpha (TNFA), and Interleukin 1-beta (IL1B); chemokines such as Interleukin 8 (IL8). They regulate the inflammatory responses by attracting and triggering immune cells. Cytokines are regulatory proteins, secreted by cells of the immune system regulating cell to cell signaling, acting through a paracrine and/or autocrine fashion [49]. Endometrial cells coming into contact with the inseminate are triggered to synthesize and release cytokines [49]. Also, the cytokine expression is a component of the inflammatory response to semen involves chemotaxis of PMNs [45]. They are regulated by receptor expression, receptor antagonists, binding proteins and by other cytokines that exert opposing effects. Interleukins regulate the interaction between lymphocytes and other leucocytes. Interferons are glycoproteins synthesized in response to virus infection, immune or chemical stimulation [49].

The TNFA is one of the key mediators of inflammation which involved in many inflammatory conditions. IL1B is a pro-inflammatory cytokine that plays a central role during pain, inflammation and is critical for the host response to pathogens. Pro-inflammatory IL8 which is produced by epithelial cells and neutrophils is responsible for the chemotaxis of neutrophils [49].

The rapid inflammatory response to insemination includes secretion of cytokines into the uterine lumen in pigs [67]. In mares, intrauterine infusions of seminal plasma, phosphate-buffered saline, skim milk extender egg yolk based extender increased mRNA expressions of *IL1B*, *IL6*, *TNFA* and *COX2* in endometrial biopsies at 12 h [68]. Interestingly, in swine, the sperm decreased the mRNA expression of *TNFA* and *IL8* at 3 h after AI, while seminal plasma and semen extender increased the mRNA expressions of *TNFA*, *IL10*, and *TGFB* at 3 h after AI [69]. In mares, endometrial biopsies showed a higher expression *IL8*, 24 h after AI [70]. In bovine uterine epithelial cells, sperm up-regulated the mRNA expressions of *TNFA*, *IL1B*, and *IL8* [24].

4.2. Immunity to sperm in the oviduct

The oviduct delivers the optical situation for fertilization, oocyte and sperm survival, sperm capacitation and embryonic development. It furnished with an active immune system that plays a

double role by attacking the ascending microbes while supporting the survival of allogenic sperm and semi-allogenic embryo [71]. The oviduct does not respond to insemination with and infiltration by PMNs [72]. Bovine oviduct epithelial cells provide an anti-inflammatory immune response under physiological conditions and the binding of that sperm-epithelial cell strengthens this response [73]. In mice, sperm in the oviduct induce transcriptional alteration with a modification in the biochemical environment in the oviduct due to the expression of novel genes [74]. In porcine, the arrival of sperm and oocytes into oviduct, induced an alteration in the oviductal gene expression, which led to modulation in the oviductal fluid composition *in vivo* and it was considered as a gamete recognition system within the oviduct [75]. In rabbits, expressions of oviductal epithelium cell surface proteins were directly influenced by semen and some proteins; structural reorganization of oviductal epithelial cells was changed within 1 to 2 h after insemination [76].

5. Molecules involved in sperm-FRT interaction.

Molecules involved in the sperm-oviduct interactions studied intensely when compared to sperm-uterine interactions. Carbohydrate moieties on the surface of the oviductal epithelium play a role in the interactions with the sperm. Annexin proteins A1, A2, A4, and A5 have been identified as oviductal receptors for bull sperm [35]. They have been shown to interact with sperm surface proteins and to be immunolocalized to the surface of the oviductal epithelium, and antibodies to annexins reduced sperm binding [77]. A sperm protein was identified as Binder of Sperm (BSP1) [35]. BSP1 in sperm increases the sperm binding to explants of oviductal epithelium [78]. Further, BSP3 and BSP5 enhance sperm binding to oviductal epithelium [79]. Another protein, beta-defensin, increases the binding of cynomolgus macaque sperm to oviductal epithelium [80].

There is a lack of studies that expose about molecules involved in sperm-uterine interactions. A recent study in bovine, revealed that bovine endometrial epithelial cells respond to sperm through TLR2/4 signal transduction, which are the membrane-spanning proteins with extracellular domains of leucine-rich repeats [81].

6. Objectives of the study

I hypothesized that the site-specific bull sperm attachment to bovine endometrium affects uterine inflammatory responses (Fig. 3).

The general objective of the study was to investigate the site-specific bull sperm attachment to bovine endometrium that affects uterine inflammatory responses.

The specific objectives of the current study were:

1. To develop a bovine *ex vivo* explant culture model to investigate the dynamics of sperm-uterine interactions *in vivo*.
2. To evaluate the effects of sperm-uterine interactions on inflammatory responses *ex vivo*.
3. To find the mechanism involved in sperm-uterine interaction.

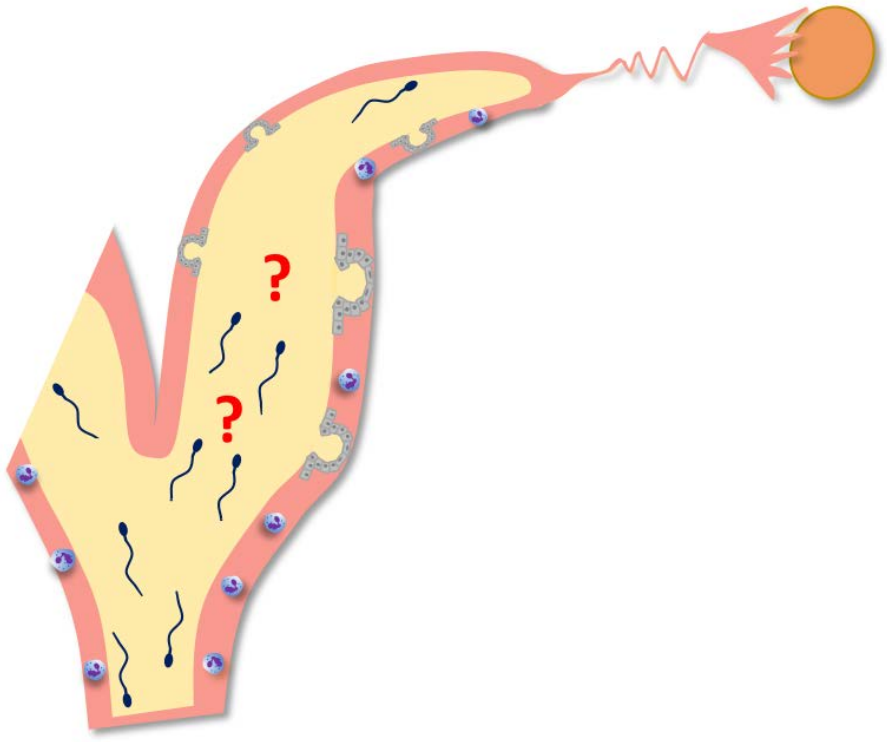

Figure 3. Illustration showing the hypothesis of the study; site-specific bull sperm attachment to bovine endometrium affects uterine inflammatory responses.

Chapter 1

Developing a bovine *ex vivo* explant culture model to investigate the dynamics of sperm-uterine interactions *in vivo*

1. Introduction

In bovine, after natural mating or artificial insemination (AI), plenty of sperm released into the uterus in which only several passes into the oviduct to reach their goal; female gamete. In the course of sperm journey from the uterus to fertilizing site, they need to interact with many portions of the female tract as well as retain in specialized regions of the tract. Sperm interaction with the female tract is very complex and involves many cell and tissue types. In bovine, the interaction, transport, and survival of sperm with the oviduct [82, 83] and utero-tubal junction [84] have been studied extensively but rarely investigated in the uterus. Nevertheless, sperm behavior in the uterus has been investigated in other species mostly in dogs [26, 30, 31] bats [28], pigs [18], and mouse [85].

In bovine, sperm interactions with the uterus have been studied rarely. We have recently shown that sperm bind to bovine uterine epithelial cells (BUEC) using a culture model of bovine sperm–uterine epithelial cells. However, *in vivo*, sperm interactions with the uterus depend on many factors. Sperm interactions with the uterus could be modulated by the mucus secretions that coat the endometrial surface, as well as by the subepithelial connective tissue. Furthermore, they could depend on the surface epithelium and/or glandular epithelium. Therefore, the current study was aimed to develop an *ex vivo* explant culture model to investigate the site and dynamics of sperm interactions with uterine epithelium under conditions that more closely resemble those *in vivo*.

2. Materials and Methods

2.1. Ethics statement

Experiments were carried out at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All experimental procedures involving animals were approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 27-74).

2.2. Reagents and media

Chemicals were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) unless otherwise stated. A modified Tyrode balanced salt solution, Tyrode Albumin Lactate Pyruvate (TALP) [86] 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 $NaHCO_3$, 0.39 mM NaH_2PO_4, 10 mM HEPES free acid, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25.4 mM sodium lactate, 0.11 mg/ml sodium pyruvate, 5 µg/ml gentamycin and 6 mg/ml bovine serum albumin with a pH of 7.4. TALP was equilibrated in a 38.5˚C incubator with 5% CO_2 in humidified air before use. Saline was used as a transport medium for uterine horns and consists of 154 mM NaCl, 10 µg/mL penicillin-streptomycin (Gibco, Grand Island, NY, USA) and 10 µg/mL amphotericin B (Gibco, Grand Island, NY, USA) with a pH of 7.4.

2.3. 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 (contra-lateral uterine horns) 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 follicular diameter according to a protocol described previously [87]. The ipsilateral horns relative to ovary containing the mature follicle of the pre-ovulatory phase (days 19-22) (Fig. 4A) and the ipsilateral horns relative to 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 [88] with major modifications. Using an 8 mm biopsy punch (Kai industries co., Ltd, Oyana, Japan) (Fig. 4B) explants were dissected from the intercaruncular endometrial regions because only intercaruncular endometrium contains endometrial glands [2] which are functional units that play a major role in secretion and distributed throughout the endometrium. Afterward, with the aid of surgical scissors, approximately 2 mm of mucus intact endometrium was extracted from the underlying tissues. The endometrial explants were gently handled to avoid the disruption of the normal anatomical structure of the endometrium and also to maintain the mucus coating to mimic the *in vivo* situations. During the preparation of explants, the TALP containing a glass petri dish was kept at 38.5˚C by a temperature-controlled micro warm plate (KM-2, Kitazato Corporation, Japan). Once collected, the explants were immediately placed in TALP. Explants were oriented as epithelial surface uppermost. Then the explants were pre-incubated in a 38.5˚C incubator with 5% CO_2 in humidified air for 15 min.

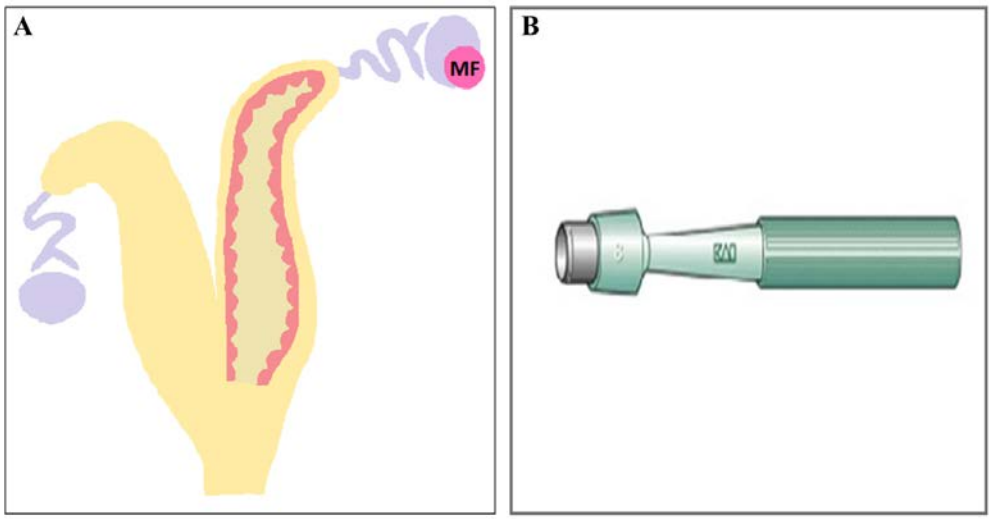

Figure 4. A. The diagram showing the ipsilateral horn relative to ovary containing the mature follicle of the pre-ovulatory phase bovine reproductive tract. **B.** 8 mm diameter biopsy punch.

2.4. Sperm sample preparation

Fresh semen samples of highly fertile Holstein bull were obtained from Genetics Hokkaido Association, Hokkaido, Japan. Fresh semen was diluted in egg-yolk extender (1:1) in a 15 mL falcon tube. The semen containing tube was maintained in a temperature-controlled heat jacket (15˚C) during transport to the laboratory (1 h) (Fig. 5A). Then the semen was maintained at 15˚C until the beginning of the experiment (6 h) (Fig. 5B). Prior to the experiment sperm was washed by diluting 2 mL of semen with 3 mL of TALP, centrifuging at 170 g for 10 min (25˚C), resuspending the pellet in 5 mL TALP and repeating twice the centrifugation. The resulting sperm pellet was re-suspended in TALP. Sperm concentration was determined using a hemacytometer (C-chip, NanoEnTek, Inc., Korea) and adjusted to 10^6 cells/mL for incubation with endometrial explants.

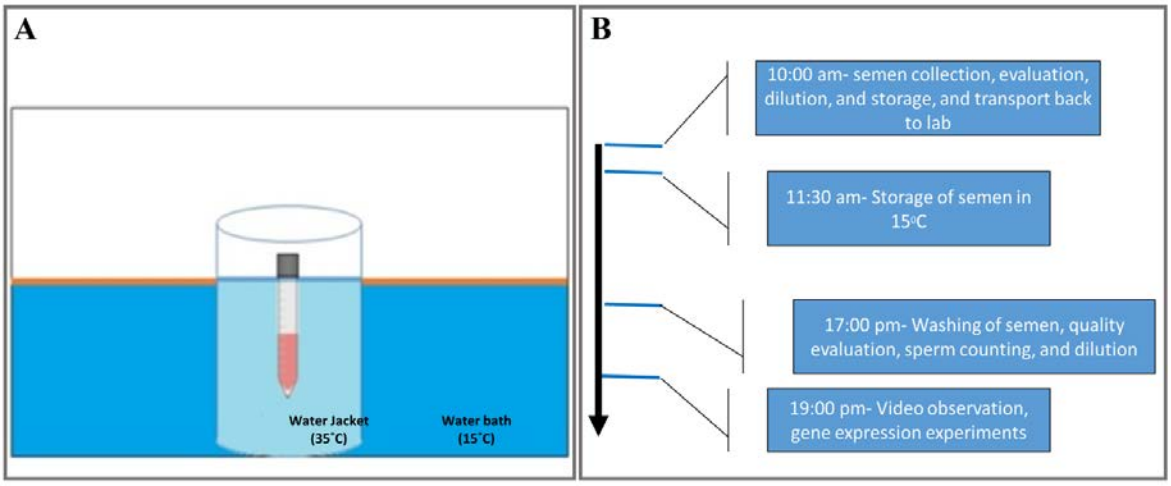

Figure 5. A. The temperature-controlled heat jacket during transport of fresh semen to the laboratory. **B.** Timeline of semen preservation until the beginning of the experiment.

2.5. Incubation of sperm with bovine endometrial explants

To investigate the time-dependent response of endometrial explants to fresh sperm, the pre-ovulatory phase explants were incubated with 10^6 cells/mL sperm for 0, 0.5 and 2 h in a 38.5˚C incubator with 5% CO_2 in humidified air using 24 well plates with 0.5 mL TALP/well. We selected this concentration because the same concentration causes effective outputs in our previous studies using BUECs [24, 81]. Meanwhile, it seems to be effective in sperm entering in glands as well as in triggering the genes. Explants without sperm served as controls for each time point. The experiment was 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 to get rid of sperm and kept in TRIZOL (Invitrogen, Carlsbad, CA, USA), and stored in -80˚C until mRNA extraction. After washing the explants in TALP, still, some sperm retained with explants. But mRNA expression of the selected genes in sperm alone is negligible [24].

2.6. JC1 sperm labeling

Sperm distribution and attachment behavior to endometrium were visualized by staining with the mitochondrial stain JC1 (AdipoGen Corp., San Diego, CA, USA). To see the sperm on the epithelial surface of explants 0.5 mL of 10^6 cells/mL sperm were pre-incubated with 6.4 µM JC1 in a 38.5˚C incubator with 5% CO_2 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 dilution of the JC1

dye in the explant culture medium provided us with helpful faint images of the epithelial surface without obscuring the view of sperm.

2.7. Observation of JC1 labeled sperm behavior after incubation with endometrial explant

To visualize the sperm behavior of fresh sperm on pre-ovulatory phase endometrial explants, JC1 labeled sperm were incubated with pre-incubated explants for 5 min, 15 min, and 30 min. For this, JC1 labeled sperm were incubated with pre-incubated explants for 15 min. Endometrial explants were oriented as epithelial surface uppermost. Before the observation, each explant was rinsed through wells containing 38.5˚C TALP and transferred to a well which contained 0.5 mL of 38.5˚C TALP, the explants oriented as epithelial surface downward. Sperm behavior in endometrial explants was viewed using an all in one fluorescence microscope (Keyence, BZ-X800, Keyence Corporation, 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 focusing was adjusted to count and visualize all sperm which entered and remained in the glands and gliding sperm through the surface epithelium.

2.8. Histology

The experiment was repeated four times using endometrial explants from four different uteri. Explants were gently washed in TALP and fixed in phosphate-buffered (PB) 10% formalin. After 24 h, the fixed tissue samples were transferred to 70% ethanol, then dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin, and cut serially in 4 µm thick slices. The endometrial sections were placed on an aminopropyl-triethoxy-silane- coated slides (S8226, Matsunami Glass Int. Inc., Osaka, Japan) and deparaffinized. The sections were stained with hematoxylin and eosin (HE) for observation of general histology. HE stained sections were observed using an all in one fluorescence microscope (Keyence, BZ-X800, Keyence Corporation, Osaka, Japan).

2.9. Scanning electron microscopy (SEM)

The pre-ovulatory phase endometrial explants and 10^6 cells/mL washed fresh sperm were incubated for 30 min and prepared for SEM. Explants without sperm served as controls. The above

experiment was repeated two times using endometrial explants from two different uteri. Explants were gently washed in TALP and fixed in PB 10% formalin. 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, Tokyo, Japan) at an accelerating voltage of 5 kV.

2.10. Determination of the number of retained sperm in uterine glands

The videos recorded from the co-culture of JC-1 labeled sperm and uterine explants for 15 min and 30 min were used for counting purposes. Recorded videos were converted to grayscale with adjusted brightness using ImageJ (version 1.51j8) before counting. To count the number of retained sperm in glands, three glands of equal size were selected in each experiment. Then the number of retained sperm within each gland was counted using enhanced videos.

3. Results

3.1. Presence of mucus in the endometrium

In the preovulatory phase 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 the medium.

3.2. Architecture and presence of uterine glands in preovulatory bovine endometrial explants.

Sections of bovine endometrial explants stained with HE showed that they were approximately 2 mm thick (Fig. 6A). Further, explants show normal tissue architecture without any disruptions (Fig. 6B).

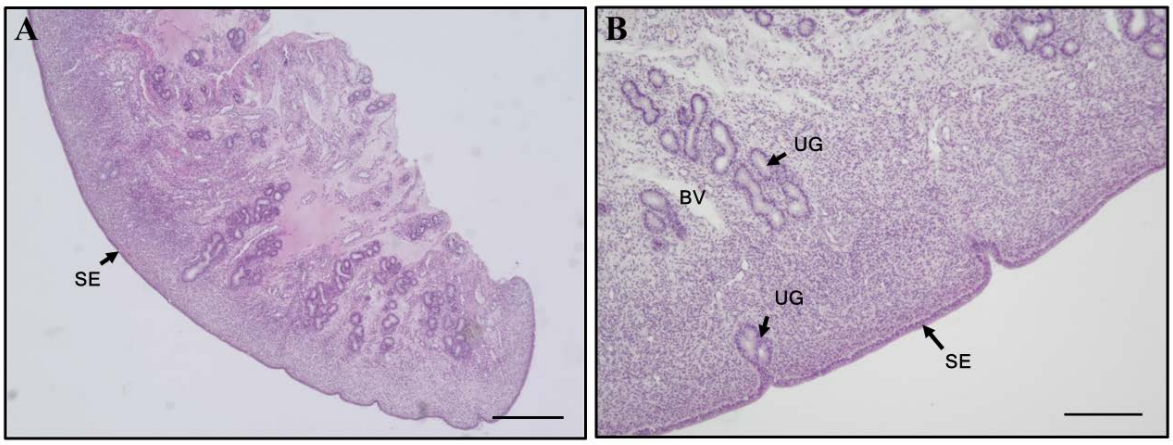

Figure 6. A. A section of an endometrial explant stained with hematoxylin and eosin. SE, surface epithelium. Bar=500 µm. **B.** Higher magnification of a section shows the uterine glands (UG) in the endometrium. BV, blood vessels. Bar=200 µm.

The surface epithelial layer was undisrupted and uterine glands were present throughout the extracted endometrium. Simple tubular uterine glands were clearly visible and maintained normal structure (Fig. 6B, 7A, 7B, 8).

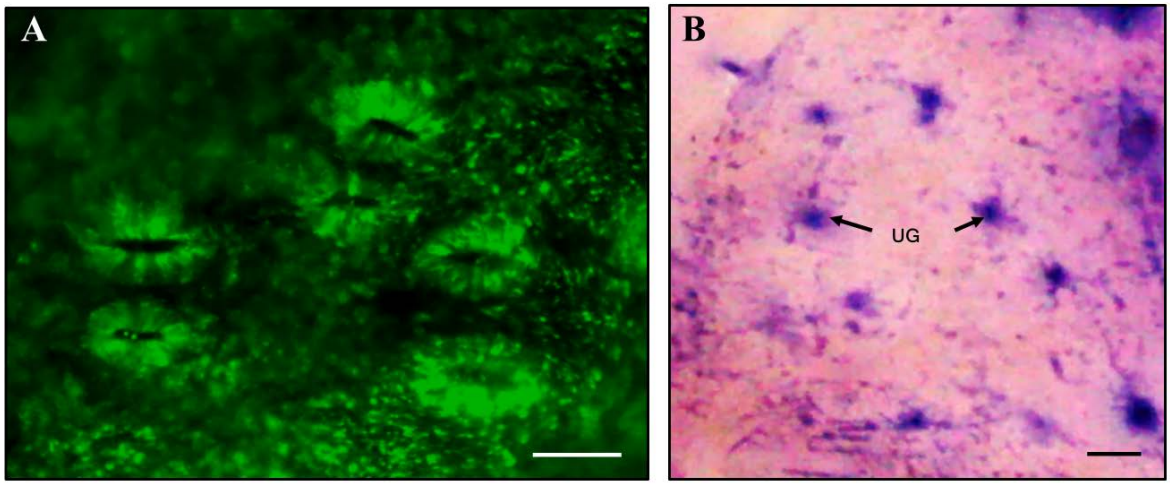

Figure 7. A. A fluorescence image shows the presence of UGs in an unstained endometrial explant, revealing autofluorescence under a BZ-X GFP (OP-87763) filter set. Bar=100 µm. **B.** A stereomicroscope image of the full thickness of a live endometrial explant stained with hematoxylin illustrates the presence of UGs (arrows). Bar=200 µm.

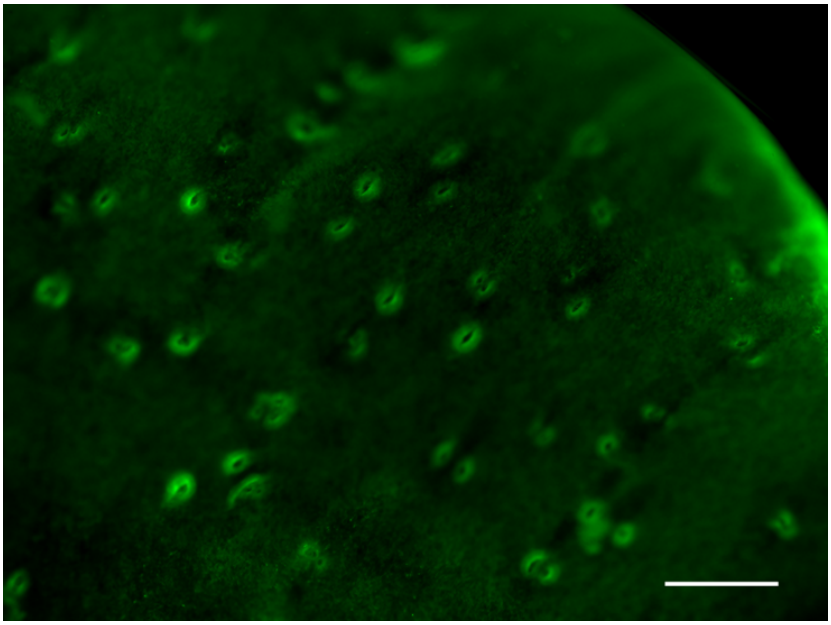

Figure 8. A fluorescence image shows the distribution of UGs in an unstained endometrial explant, revealing autofluorescence under a BZ-X GFP (OP-87763) filter set. Bar= 200 µm.

3.3. Sperm glided over the surface epithelium and entered uterine glands.

Epifluorescence video microscopy revealed that sperm glided over the surface epithelium until they encountered and entered uterine glands at 5 min (Fig. 9). Motile sperm could be seen within glands after 30 min of co-incubation (Fig. 10).

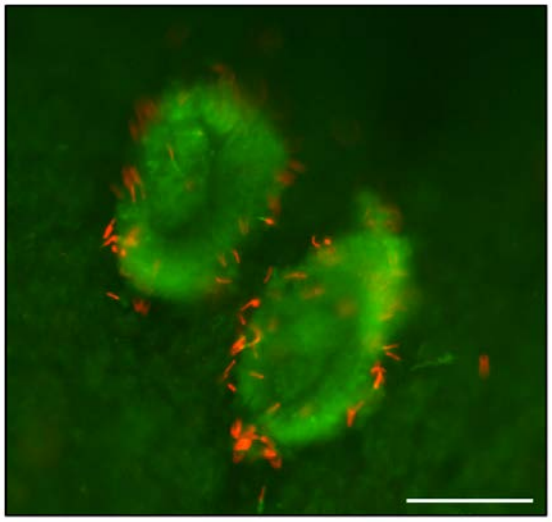

Figure 9. An epi-fluorescence image of sperm stained red in the midpiece region by the mitochondrial stain JC1. The sperm are gathered at the entrances of two UGs (green) 5 min after incubation of pre-ovulatory phase uterine explants with JC1-stained sperm. Bar=100 µm.

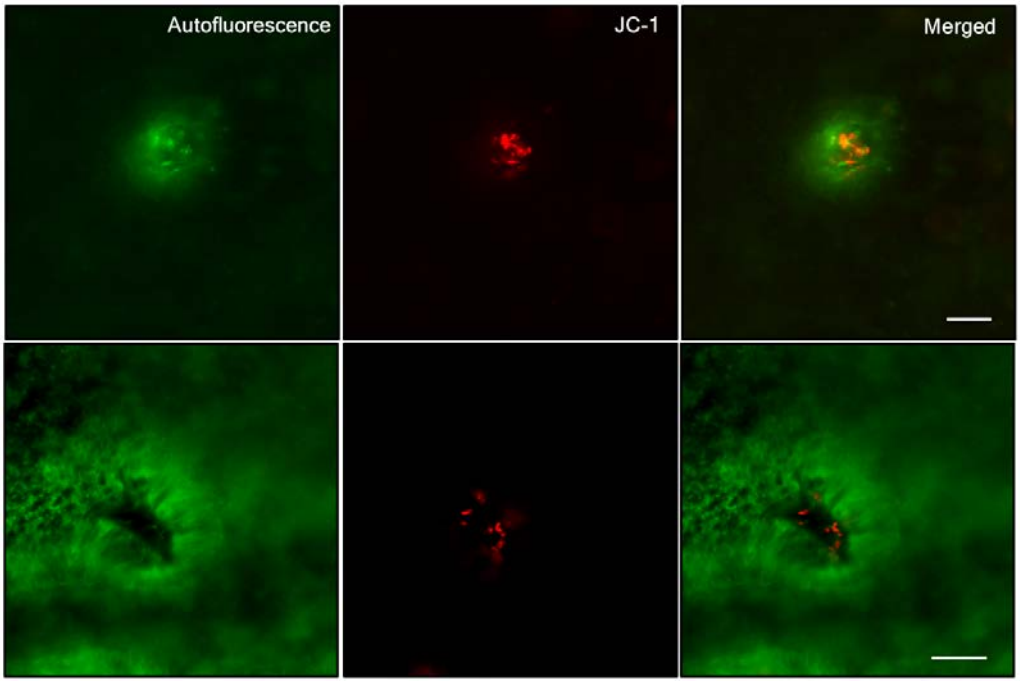

Figure 10. JC1-stained midpiece mitochondria of sperm within pre-ovulatory phase uterine glands after 30 min incubation with explants of pre-ovulatory endometrium. Separate rows are representative of independent experiments. Bars=50 µm.

3.4. SEM revealed the presence of uterine glands and sperm in glands

SEM images clearly indicated the presence of uterine glands opening to the uterine lumen (Fig. 11). SEM showed that the sperm were retained within uterine glands in distinct clusters (Fig. 12B). Sperm were not attached to the surface epithelium (Fig. 12A). Retained sperm heads were contacted with the glandular epithelium (Fig. 12B) and projections of the sperm tails from the openings into the uterine lumen were clearly identified (Fig. 12A).

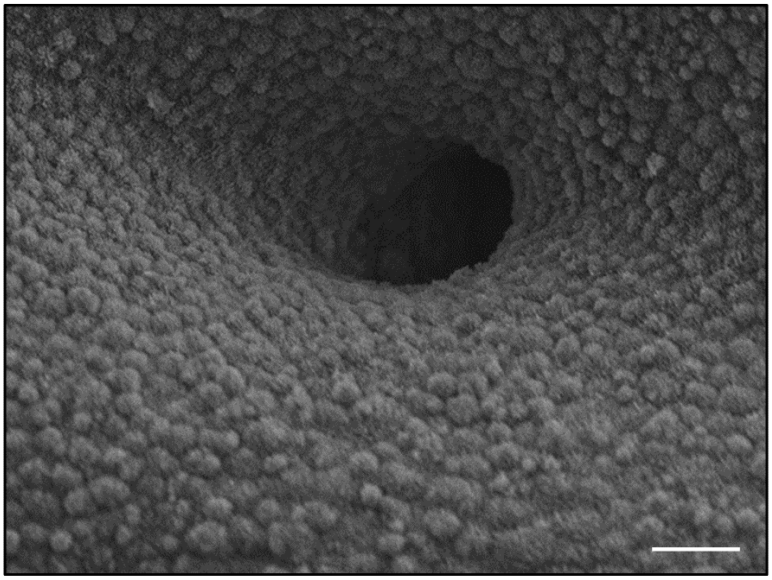

Figure 11. SEM micrograph shows the UG opening to the uterine lumen. Bar=10 µm.

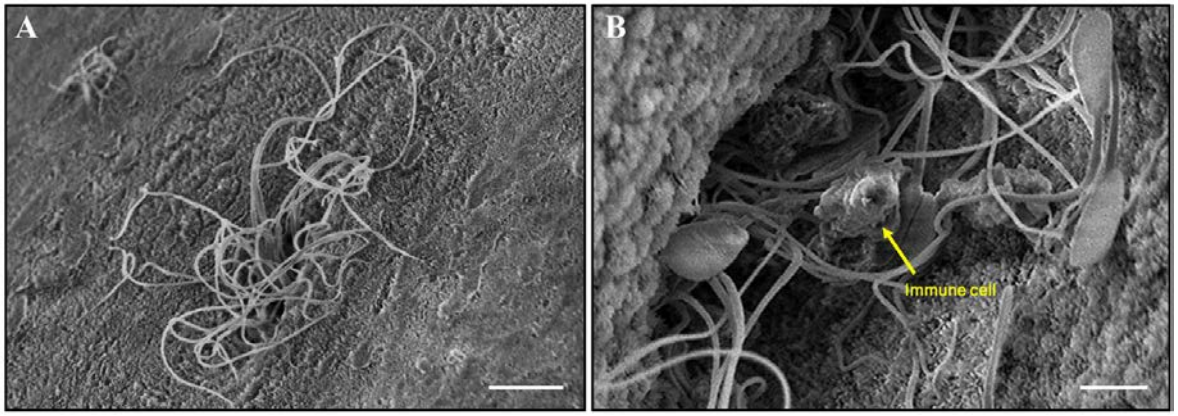

Figure 12. Scanning Electron Microscopy. **A.** Sperm appear only within or on the rims of uterine glands (UGs) and not attached to surface epithelium. Sperm tails are visible in the UGs. Bar=10 µm. **B.** Immune cells and sperm within a UG. Bar=5 µm.

3.5. Number of sperm in uterine glands

The number of retained sperm cells within the uterine glands was 15.5 ± 1.7 and 13.4 ± 1.8 sperm/gland at 0.25 and 0.5 h respectively. There is no significant difference in sperm cell number per gland at 0.25 and 0.5 h (Fig. 13).

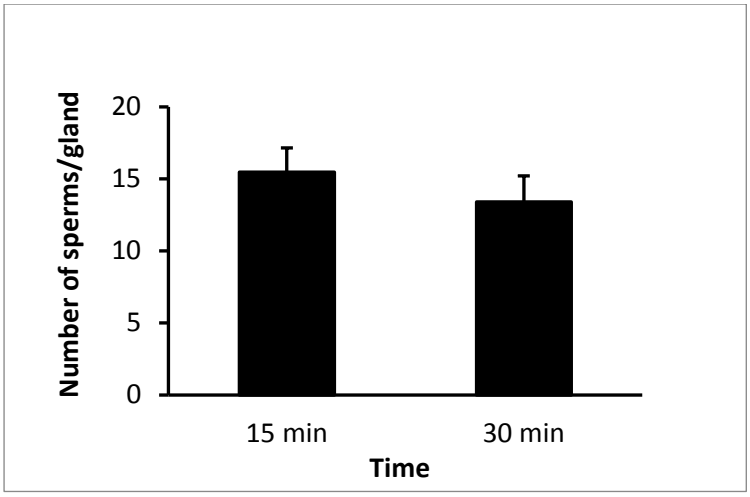

Figure 13. SE_M number of sperm within pre-ovulatory phase uterine glands at 15 min and, 30 min of co-incubation. Data are presented as mean ± SE_M of five independent experiments. Three glands were counted per group per experiment.

4. Discussion

Observations using an *ex vivo* explant culture model of uterine endometrium and fresh sperm provide the first 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 [89, 90]. 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.

There is a lack of studies, which detailed sperm interactions with the bovine uterus. Recently, our group studied the frozen-thawed bull sperm interactions with bovine uterine epithelial cells using a cultured cell culture model. Since, *in vivo*, sperm interactions with uterus depends not only on epithelial cells but on many other factors, we developed an *ex-vivo* culture model. Initial observations revealed that uterine explants show normal anatomical structure and sperm retained within the uterine glands.

Sperm retention in the uterine glands has been reported in many animal species, such as guinea pig, stoat, hedgehog, mole [25], dog [26], sow [18], rabbit [27] and bat [28]. But rarely investigated in the cow. Few sperm were found in uterine glands of a cow 24 h after AI [27]. This is the only previous report that mentioned about sperm retention in bovine uterine glands. Therefore, the current study, detailed sperm retention in bovine uterine glands using the *ex vivo* culture model.

Investigation revealed that sperm glide over the surface epithelium and enter uterine glands. Immediately after co-incubation, we observed the sperm midpieces which glide over surface epithelium and enter specific areas which are the uterine glands. At 30 min the majority of the sperm is retained within the uterine glands. Also, it is visible that sperm did not attach to surface epithelium but only retained within the uterine glands. This observation further supported by SEM images where sperm retained only in the uterine glands but not in the surface epithelium. The SEM observations show that sperm heads were located in the uterine glands and only the tails of the sperm were clearly seen. From the obtained SEM photos it seems that sperm bound with their heads to the glandular epithelium. Furthermore, sperm-epithelium binding seems to be strong because even after washing they retain in the uterine glands. This is supported by a previous study, where sperm were detected in the uterus by histology and SEM but not by flushing [30].

Similar observations were found in previous studies in other species. In the rabbit, sperm appeared in the uterine glands and fewer sperm were found in the uterine lumen 2 h after natural mating [27]. Sperm were found in sow uterine glands after natural mating only in the pre-ovulatory phase where sperm were clumped together with heads aggregated in parallel rows around some central masses. The clumps were located most frequently near the glandular opening. Most of the glandular openings were completely filled with a large number of sperm [27]. Histological observations showed that sperm were located in the uterine glands in a cow 24 h after AI, but penetration of sperm into the uterine glands was not observed by SEM. Most sperm heads were directed toward the distal end of the uterine glands [27]. Sperm were mainly clustered in the uterine glands, most frequently in the neck of these glands following natural mating in dogs [26]. The sperm which were found in the uterine lumina may the gliding ones over the endometrial surface which is evident from our observations.

The number of retained sperm within the uterine gland was 15.5 and 13.4 sperm/gland at 0.25 and 0.5 h respectively after sperm incorporation. A study in the dogs revealed that after 24 h the

majority of the uterine glands with more than five sperm when inseminated during the ovulatory phase. Meanwhile, the uterine glands mostly contained one sperm were frequently found when inseminated during the pre-ovulatory phase [30]. However, the percentage of uterine glands with sperm 24 h after natural mating in dogs was higher [26]. This deviation from our results in the number of sperm per gland may be due to the species and/or time variation.

There are few additional factors that may cause sperm to be retained in uterine glands, such as the narrow region, [83] secretions and chemotaxis to chemicals from the glands. There are some findings related to chemotaxis of sperm such as follicular fluid stimulates chemotactic activities of human sperm [91], rabbit eggs exert a chemotactic influence on rabbit sperm [92] and chemotaxis of sperm by egg white and ovarian cyst fluid [93]. Therefore, there is a possibility of some chemoattractants from the uterine glands which attract the sperm since histotroph from glands contains many factors such as growth factors, cytokines, hormones, a variety of enzymes, glucose, adhesion molecules and transport proteins [5, 94, 95, 96, 97].

The uterine explants were collected only from intercaruncular endometrial regions because only intercauncular endometrium contains endometrial glands [2] which are functional units that play a major role in secretion and distributed throughout the endometrium. The uterine explants were gently handled to avoid the disruption of the normal anatomical structure of the endometrium and to maintain the mucus coating. For video observation JC-1 mitochondrial stain was used. Although mitochondria in the epithelia were also stained by the JC-1, the midpieces of the sperm stained much more brightly and were readily distinguished from the epithelial cells.

In the present study, three times washed fresh sperm at the concentration of 10^6 cells/mL was used. This concentration was selected because the same concentration causes effective outputs in our previous studies using BUECs [24, 81]. Meanwhile, it seems to be effective in sperm retention in glands.

It can be concluded that the developed bovine *ex vivo* explant culture model can be successfully used to investigate the dynamics of sperm-uterine interactions. Further, the observations, obtained by *ex vivo* explant culture model provides evidence that uterine glands serve as a site where sperm interact with glandular epithelium.

Chapter 2

Evaluating the effects of sperm-uterine interactions on inflammatory responses *ex vivo*

1. Introduction

In the course of migrating through the female tract to the egg, mammalian sperm interact closely with the epithelium lining the tract [35, 98]. Most work in the past 20 years has focused on 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 [82, 83]; 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 the uterus to receive embryos [12, 14].

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 [20, 62]. In addition, the bovine uterus has a well-developed innate immune system that can mount an inflammatory response to sperm [99] as well as to pathogens [100]. The inflammatory response to sperm contribute 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; i.e, production of anti-sperm antibodies that could reduce the fertility of the female [101, 44].

Bull sperm are rapidly removed during the inflammatory response mainly by PMNs [14]. The PMN influx after semen deposition is essential not only to remove sperm but also to remove pathogens that may be introduced during mating [14, 59]. An influx of PMNs into the uterine lumen to remove sperm and pathogens has also been reported in horses [56] and, pigs [48]. 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 [102, 59].

We recently reported that sperm binding to monolayer cultures of bovine uterine epithelial cells (BUEC) induces an acute inflammatory response in the epithelium [24]. However, *in vivo*, sperm interactions with the immunological defenses of the uterus could be mediated by surface epithelium and/or glandular epithelium. Therefore, the developed bovine explant culture model was used to investigate sperm behavior and immune responses. Initial observations prompted to hypothesize that sperm interact with the glandular epithelium of the preovulatory uterus to trigger immune responses. To test this hypothesis, the site of sperm interactions was identified that links to immune responses.

2. Materials and methods

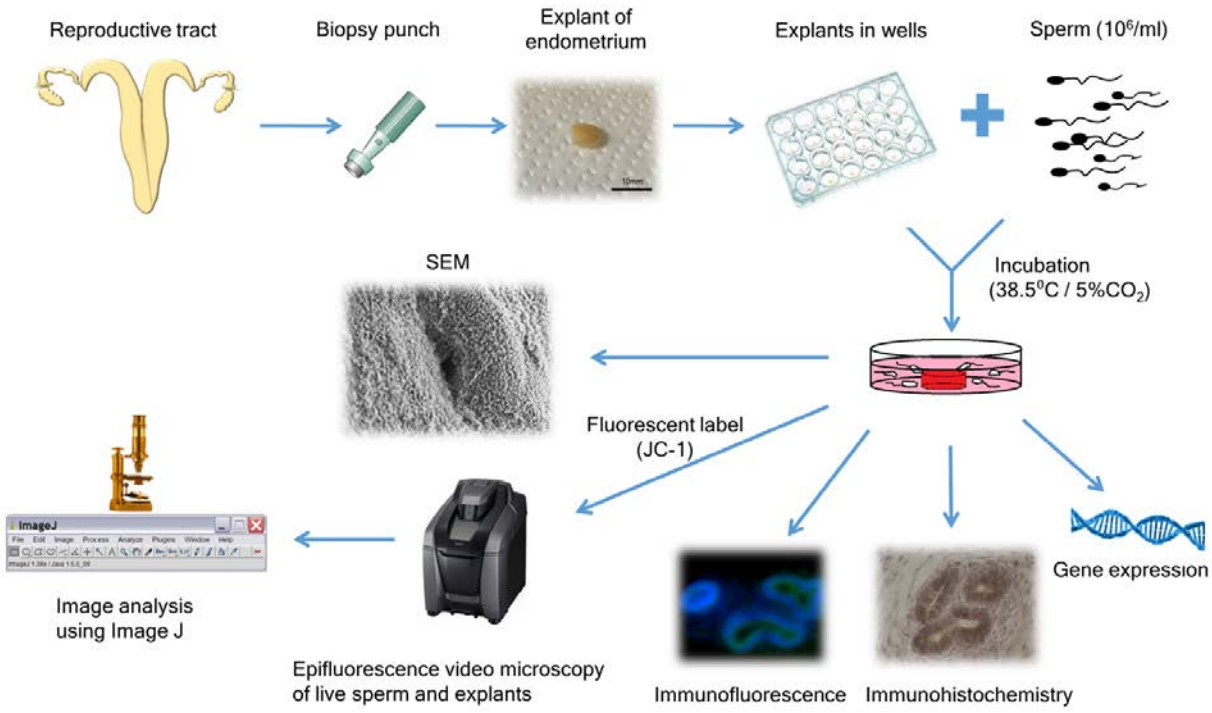

Figure 14. A diagram showing the methodology of the study.

2.1. Incubation of sperm with bovine endometrial explants

To investigate the time-dependent response of endometrial explants to sperm, pre-ovulatory phase explants were incubated with 10^6 sperm/mL for 0, 0.5 and 2 h in a 38.5˚C incubator with 5% CO_2 in humidified air using 24 well plates with 0.5 mL TALP/well. Explants without sperm served as controls for each time point. Heat-inactivated sperm was prepared by incubation of washed fresh sperm in a water bath at 56˚C for 30 min [103]. To investigate the effect of heat-inactivated sperm, the pre-ovulatory phase explants were incubated with 10^6 heat-inactivated sperm/mL for 2 h. To investigate the effect of sperm on luteal phase explants, the luteal phase explants were incubated with 10^6 sperm/mL for 2 h. Explants without 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, Carlsbad, CA, USA), at -80˚C until mRNA extraction.

2.2. Isolation of PMNs from blood

PMNs were isolated from whole bovine blood from three animals according to a protocol described previously [71]. 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 NH_4Cl, 3.4 mM $KHCO_3$, and 96.7 µM EDTA) for 10 sec and centrifuged at 500 g for 10 min at 10˚C to purify PMNs from red blood cells. Finally, the isolated PMNs were washed twice with PBS.

2.3. Identification of PMNs in uterine glands along with the sperm using SEM

After incubation of uterine explants and sperm, the explants were prepared for the SEM as described previously. Briefly, the pre-ovulatory phase endometrial explants and 10^6 cells/mL washed fresh sperm were incubated for 30 min and prepared for SEM. Explants without sperm served as controls. Both the above 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, Tokyo, Japan) at an accelerating voltage of 5 kV.

The number of PMNs per uterine gland in endometrial explants incubated for 30 min with and without sperm were counted using the SEM images.

2.4. 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 [104]. The concentration and purity of extracted RNA were determined using NanoDrop Spectrophotometer (2000c, Thermo Scientific, Waltham, MA, USA) by the absorbance at 260 nm and the 260/280 nm ratios, respectively. After measurements, the RNA was stored at -80˚C in RNA storage solution (Ambion, Austin, TX, USA) until cDNA synthesis.

The cDNA was synthesized following a protocol described previously [73] with minor modifications. First, a DNase treatment step was performed using RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) to remove residual genomic DNA and other contaminants. Through which, 1 µg of 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 of RQ1 RNase-free DNase 10X Reaction Buffer, 2 µl of RQ1 RNase-free DNase, and Nuclease-free water (Invitrogen, Carlsbad, CA, USA) to a final volume of 10 µl followed by addition of 1 µl of the 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 of 10 mM PCR Nucleotide Mix (dNTP) (Roche Diagnostics, Indianapolis, IN, USA) 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 0.1M 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, Tokyo, Japan) using SYBR Green PCR Master Mix (Bio-Rad Laboratories, USA). Simply, a total 10 µl reaction mix consisting of 2 µl/sample synthesized cDNA, 5 µl of QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany), 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-sec denaturation at 95˚C, 15-sec annealing at 55-58˚C, and 30-sec extension at 72˚C). A negative control, which was prepared by

replacing the cDNA sample with nuclease-free water, was kept in each run. The primers 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 gene expression were determined using the Delta-Delta comparative threshold method [105]. The housekeeping gene, *β-actin*, was used as an internal standard for 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)
B-actin	Forward	TCACCAACTGGGACGACATG	NM_173979.3	51
	Reverse	CGTTGTAGAAGGTGTGGTGCC		
IL8	Forward	CCAATGGAAACGAGGTCTGC	NM_173925.2	51
	Reverse	CCTTCTGCACCCACTTTTCCT		
TNFA	Forward	CAAAAGCATGATCCGGGATG	NM_173966.3	51
	Reverse	TTCTCGGAGAGCACCTCCTC		
IL1B	Forward	AATCGAAGAAAGGCCCGTCT	NM_174093.1	51
	Reverse	ATATCCTGGCCACCTCGAAA		
PGES	Forward	AAAATGTACGTGGTGGCCGT	NM_174443.2	51
	Reverse	CTTCTTCCGCAGCCTCACTT		
TLR2	Forward	CATGGGTCTGGGCTGTCATC	NM_174197.2	51
	Reverse	CCTGGTCAGAGGCTCCTTCC		

2.5. Immunohistochemistry

To investigate the effect of sperm on the pre-ovulatory 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, then dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin, and cut serially in 4 µm 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 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% H_2O_2 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-alpha primary antibody (1:400, 2.5 µg/ml, MCA2334, BIO-RAD., CA, USA) 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 µg/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% H_2O_2. 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, coverslipped, and observed with a conventional light microscope (Nikon, Microphot-FX, Japan) and images were obtained (Nikon, Digital sight DS-SM, Japan). The negative control sections omitted the primary antibody.

2.6. Immunofluorescence Analysis

Endometrial sections were fixed and blocked as described above, followed by incubation overnight at 4˚C with mouse monoclonal anti-bovine tumor necrosis factor-alpha primary antibody (1:400, 2.5 µg/ml, MCA2334, BIO-RAD., CA, USA). 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 µg/ml goat anti-mouse IgG labeled with Alexa Fluor, Invitrogen, Thermo Fisher Scientific, USA) for 30 min. Sections were washed, and coverslips were mounted using VECTASHIELD mounting medium (H-1000; Vector Laboratories, Burlingame, CA 94010, USA). The fluorescence signal was then captured using an all in one fluorescence microscope (Keyence, BZ-X800, Osaka, Japan) 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.

2.7. Statistical analysis

GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Students 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 ± standard error of the mean (SE_M). Data were considered to be statistically significant at *P<0.05, **P<0.01, ***P<0.001.

3. Results

3.1. Clusters of sperm in the uterine glands along with endogenous neutrophils.

Clusters of sperm were seen in uterine glands along with immune cells probably neutrophils (Fig. 15A, 15B). Endogenous neutrophils (Fig. 15B) and exogenous neutrophils (Fig. 15C) show similar morphological appearance and size. Therefore, it can be concluded that probably neutrophils were present along with the sperm. Sperm phagocytizing by endogenous neutrophils were observed within and above the uterine glands (Fig. 15A, 15B). Meanwhile, endogenous neutrophils cannot be visible in the absence of sperm (Fig. 15D). PMNs number was significantly higher in the uterine glands which incubated with the sperm (Fig. 16).

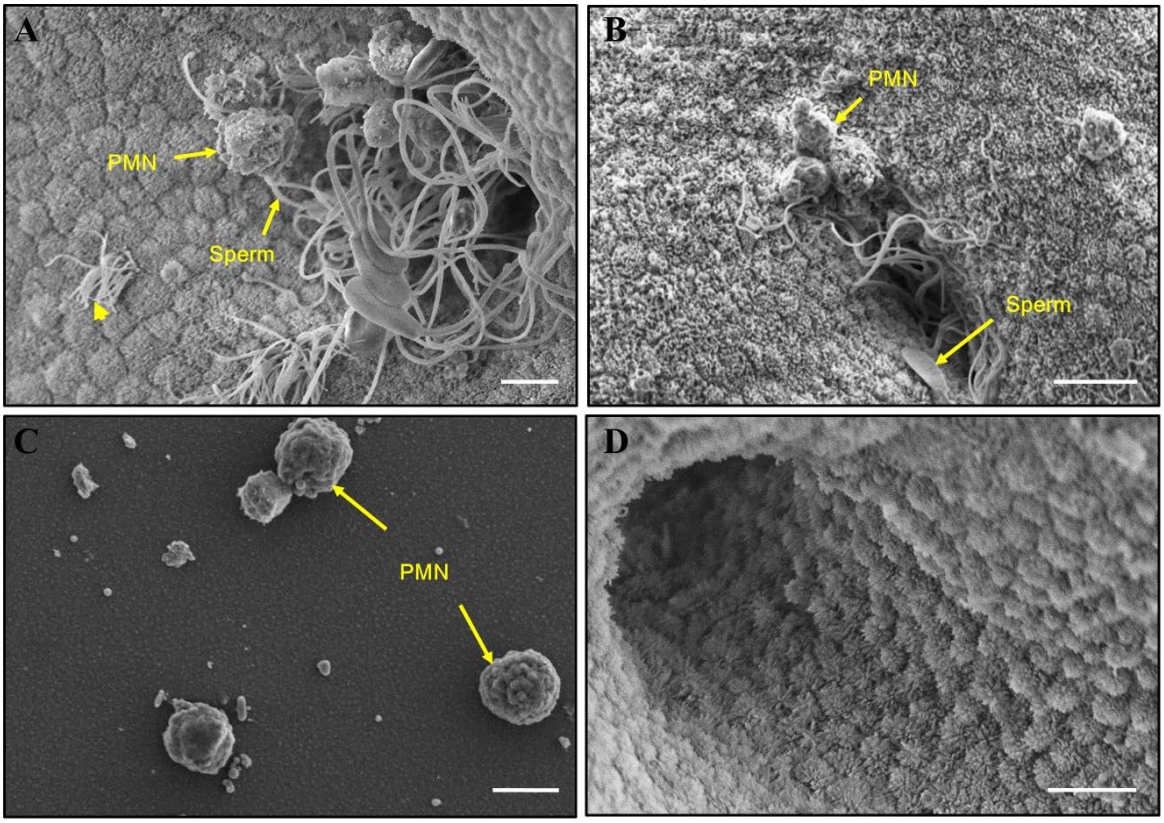

Figure 15. A. PMNs binding to sperm at the opening of a UG. Note that a single ciliated surface epithelial cell can be seen (arrowhead); the remainder of the surface epithelial cells show microvilli on their apical surfaces. Bar=5 µm. **B.** PMNs appear on the rims of a uterine gland. Bar=10 µm **C.** Bovine PMNs collected from blood. Bar=5 µm. **D.** The apparent absence of immune cells within UGs in the absence of sperm. Bar=5 µm.

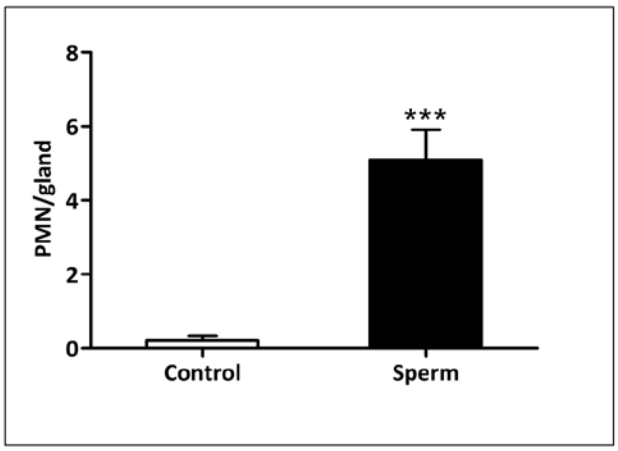

Figure 16. Mean ± SE_M number of PMNs per uterine gland in endometrial explants incubated for 30 min with and without sperm. Data are presented as mean ± SE_M of two independent experiments. Ten glands were counted per each group. ***$p<0.001$ denotes a significant difference.

3.2. Sperm up-regulated gene expression in the pre-ovulatory phase bovine endometrium.

Sperm-explant co-culture for 2 h resulted in a significant upregulation of our selected pro-inflammatory cytokines *TNFA* and *IL1B*, as well as *PGES* and *TLR2* mRNA expression, but *IL8* (a strong chemokine for neutrophils) expression started to increase much earlier at 0.5 h and continued to increase through 2 h co-incubation ($p<0.05$). (Fig. 17).

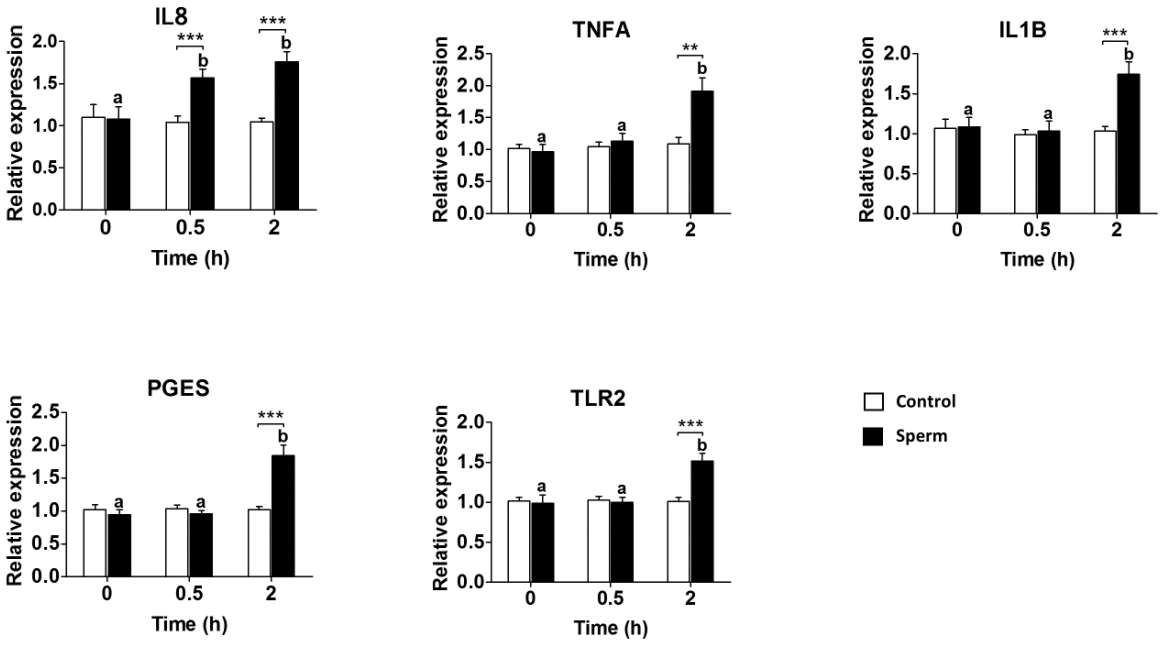

Figure 17. Time-dependent effect of sperm on the relative mRNA expression of pro-inflammatory cytokines, *PGES*, and *TLR2* in the pre-ovulatory bovine endometrial explants. Explants had been incubated with 10^6 cells/mL sperm for 0, 0.5 and 2 h. Data are presented as mean ± SE_M of five independent 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.

3.3. Sperm up-regulated the TNFA protein expression in uterine glands.

TNFA protein expression was detected using both immunofluorescence and DAB staining. Both staining methods showed that when sperm were co-incubated with uterine explants for 4 h, sperm upregulated the TNFA expression in uterine glands (Fig. 18).

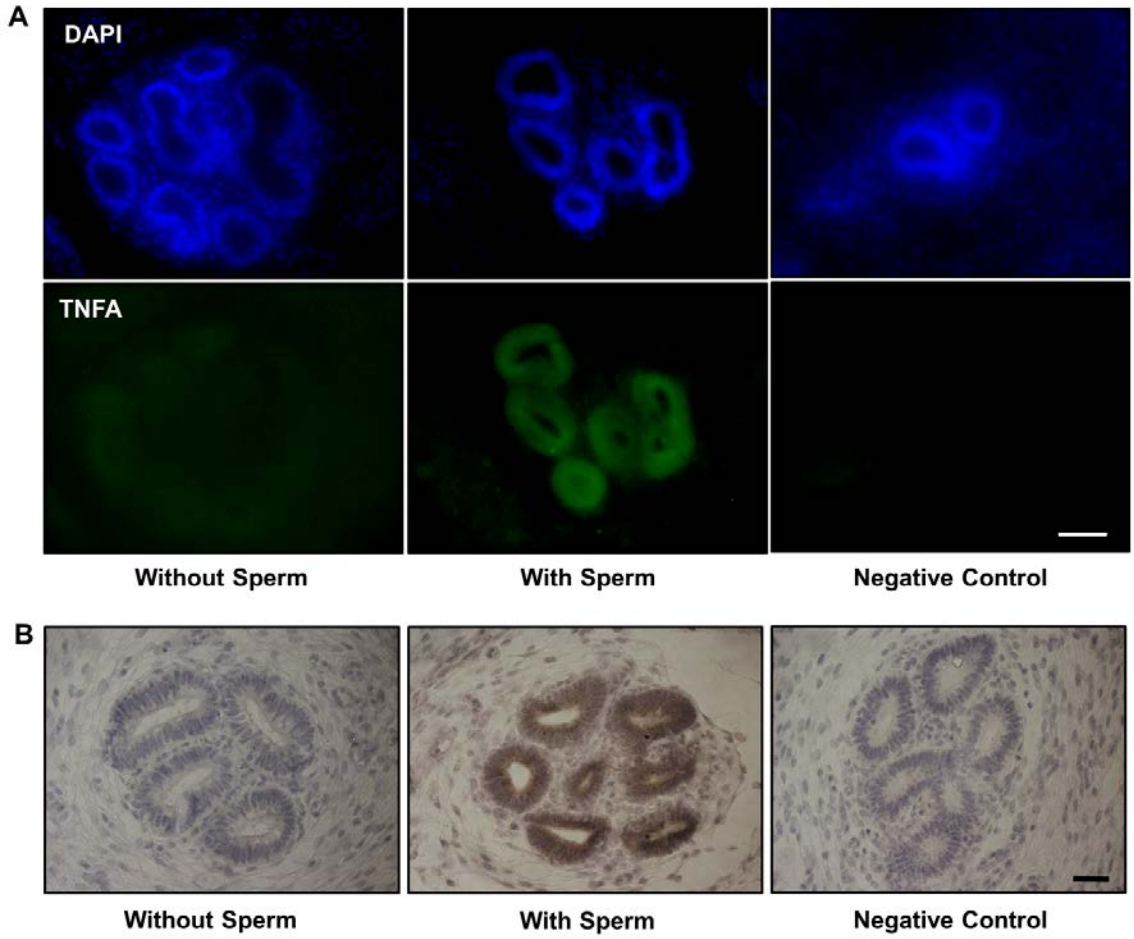

Figure 18. TNFA expression in endometrial explants, particularly in uterine glands after incubation of bovine uterine explants for 4 h with and without sperm using (**a**) immunofluorescence labeling with Alexa Fluor-conjugated anti-TNFA antibody, and (**b**) immunostaining with peroxidase-conjugated anti-TNFA antibody, visualized using DAB (brown). The primary antibody was omitted in the negative control sections. Nuclei are counterstained with hematoxylin. Bars (a) = 50µm, (b) = 20µm.

3.4. Heat-inactivated sperm neither retained in glands nor stimulate gene expression.

At 30 min of uterine explant co-incubation with heat-inactivated sperm, none of the inactive sperm were seen retained within the uterine glands (Fig. 19A). Meanwhile, heat-inactivated sperm did not affect the gene expression of *TNFA* and *IL8* (Fig. 19B).

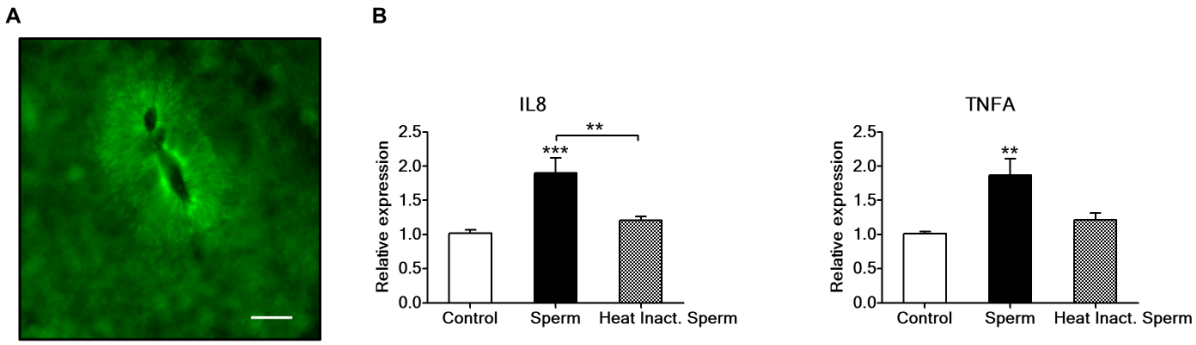

Figure 19. A. Epi-fluorescence overlay image taken using green and red filters showing the absence of heat-inactivated sperm (which would fluoresce green if present) in the pre-ovulatory phase uterine glands (which autofluorescence green). Uterine explants were incubated with 10^6 cells/mL heat-inactivated sperm for 30 min. Bar=50 µm. **B.** Relative mRNA expression of pro-inflammatory cytokines in pre-ovulatory 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 ± SE_M of five independent experiments. Three endometrial explants from individual cows were used in each experiment. **p<0.01, ***p<0.001 denote significant difference.

3.5. Sperm neither retained in glands nor stimulate gene expression in luteal phase uterine explants.

When luteal phase uterine explants were co-incubated with sperm, sperm did not retain within the glands (Fig. 3-7A), but sperm were gliding over the surface epithelium. Meanwhile, sperm did not affect the mRNA expression of *TNFA*, *IL8*, and *IL1B* in the luteal phase endometrium (Fig. 20B).

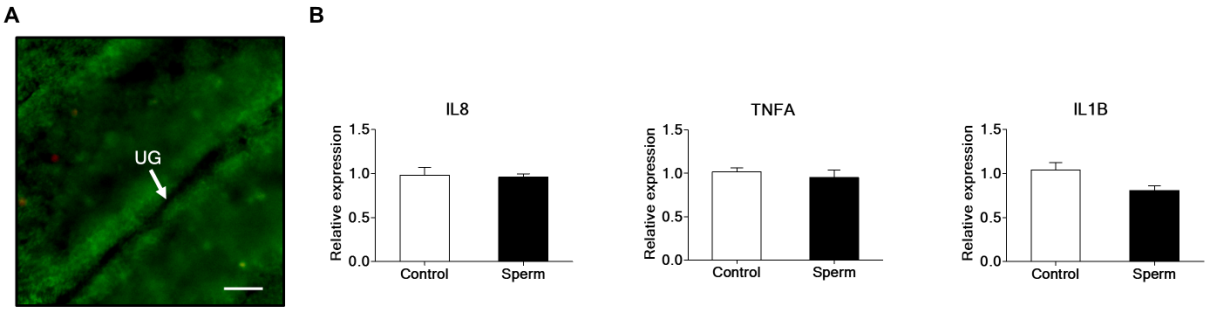

Figure 20. A. Epi-fluorescence overlay image taken using green and red filters showing the absence of sperm (which would fluoresce red if present) within the luteal phase uterine glands (which autofluorescence green). The luteal phase endometrial explants were incubated with 10^6 cells/mL sperm for 30 min. UG, uterine gland. Bar=50 µm. **B.** Relative mRNA expression of pro-inflammatory cytokines in luteal phase

bovine endometrial explants incubated with 10^6 cells/mL sperm and without (control) sperm for 2 h. Data are presented as mean ± SE_M of five independent experiments. Three endometrial explants from individual cows were used in each experiment.

4. Discussion

Observations using an explant culture model of uterine endometrium and fresh sperm provide the evidence that sperm interaction with uterus triggered the immune responses. Gene and protein expression data indicate that sperm interaction with glandular epithelium triggers local uterine innate immune responses *via* the upregulation of pro-inflammatory cytokines. Initiation of the inflammatory response led to the migration of PMNs into the glands, where they came into contact with sperm.

Sperm retention in the uterine glands has been reported in many mammalian species. 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.

Gene and protein expression data indicate that sperm interaction with glandular epithelium triggers local uterine innate immune responses *via* the upregulation of pro-inflammatory cytokines. Sperm up-regulated endometrial mRNA expression of pro-inflammatory cytokines *IL8* (at 0.5 h) and *TNFA* and *IL1B* (at 2 h). Previously, our group reported that frozen-thawed motile sperm upregulated similar genes in BUECs, in which the uterine epithelium was somewhat dedifferentiated and surface epithelium could not be distinguished from the glandular epithelium [24]. Further, in the present study, synthesis of TNFA protein was detected, which is a strong pro-inflammatory marker, in the glandular epithelium. These observations lead to propose that sperm entering glands trigger a uterine immune response that begins in the glands.

SEM images indicated that sperm bound by their heads to the glandular epithelium, as well as to each other. SEM images showed that PMNs were present in the glands. PMNs are known to rapidly enter the uterine lumen after insemination of cows [59], as well as of females of several other species of mammals [14, 52, 55, 56]. 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 [14]. 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 [54], goats [14], pigs [22], and rabbits [61]. PMN phagocytosis of sperm within uterine glands was reported in little brown bats, guinea pigs, horseshoe bats, rabbits, and pigs [25, 27, 28]. In addition to PMNs, there is some evidence that macrophages phagocytize sperm within uterine glands in rabbits and cows [27]. 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 [49].

Observations revealed that sperm entered and remained in the pre-ovulatory 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 [6, 8, 9, 106]. Although the physical attraction of sperm for surfaces would tend to direct them into glands, as discussed above, the physical and/or chemical nature of the luteal phase secretions in the glands may have prevented them from doing so. Our data is supported by a previous study where the majority of glands with sperm were observed when inseminated during ovulation or pre-ovulation period but a fewer number of glands with sperm were observed when inseminated after ovulation [30]. Another study in dogs showed that sperm attached preferentially to estrus uterine epithelium, compared to the luteal or anestrous uterine epithelium [29]. The effects of the phase of the estrus cycle on sperm retention in glands may relate to the hormone influence. Also, it is reported that estrous cycle stages related to the uterine inflammatory response [107].

The present data further reveal that viable sperm retained within uterine glands but not the heat-inactivated sperm. Meanwhile, heat-inactivated sperm did not trigger the genes. A similar result was observed in a previous study in which the cow was inseminated directly into the uterus. Within 1 h, 96% of the heat-inactivated sperm had been discharged into the vagina, while only 26% of the live

sperm had been discharged [20]. In our previous study, some active sperm attached to BUECs monolayers and heat-inactivated sperm did not attach to BUECs [24]. Also, another study in ovine claimed that dead sperm did not migrate into the cervical glands [32]. An *in vitro* study in pigs showed that viable sperm retains in *ex vivo* uterine segments [19]. This shed evidence that only the live sperm retains in the uterine glands and up-regulate the genes. Dead sperm which couldn't attach to the glands might be removed by backflow which indeed has been proven to be an effective tool in removing dead sperm [20]. It is also suggested that live sperm penetrate into the glands by their own active force but not by any external forces.

Immune cells especially PMNs were observed along with clusters of sperm in uterine glands, which may initiate sperm clearance by phagocytosis. Therefore, uterine glands may play a role as a selection mechanism for the sperm. Sperm retention in uterine glands provides a mechanism to reduce the sperm number entering the oviduct. Hypothetically, of the whole sperm population which enters the uterus, those having a fertilizing ability might travel directly to the oviducts and are possibly not recognized by the selective mechanism of the uterine gland, whereas viable sperm in less mature stage and defective sperm might retain in the uterine glands for the selective mechanism. Further, a mechanism of the female immune system in the selection of the fertilizing sperm was reported in rabbits. A secretion of immunoglobulins (IgG) into the lumen of the tract in association with the leucocytic reaction and thereby IgG-coated sperm apparently removed from the tract such that sperm not bound to antibodies become the fertilizing sperm and concluded that the fertilizing sperm population is derived from those cells which first enter the oviduct after mating, and have thereby avoided IgG-mediated sperm removal [108]. Therefore, a similar mechanism may be involved in selecting the fertilizing sperm in bovine too. Therefore, further investigations needed in order to find the mechanism which describes the attraction and selection of sperm by uterine glands.

It can be concluded that the developed bovine explant culture model can be used to investigate the initial sperm-uterine interactions and immune responses (Fig. 21). Here, the culture model provided evidence that sperm entry into uterine glands triggers the innate immune response. Further it shed evidence that uterine glands serve as a site where sperm interact with glandular epithelium to trigger the innate immune response to rapidly clear sperm from the uterus and thus prepare the endometrium for embryo implantation.

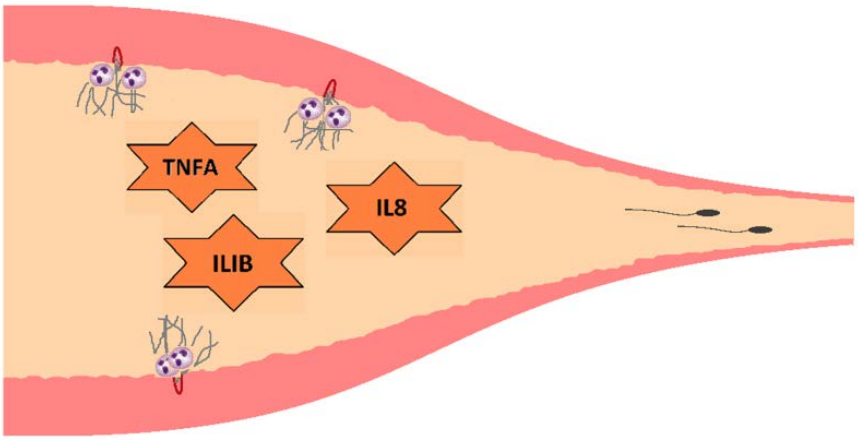

Figure 21. Diagram showing that sperm entry into uterine glands triggers the innate immune responses.

Chapter 3

The mechanism involved in sperm-uterine interaction

1. Introduction

The uterus involved in many important physiological events such as implantation, placentation, and maintenance of pregnancy. During these periods, the uterus encounter with invading pathogens, allogenic sperm, and semi-allogenic embryo. Sperm-uterine interactions have been shown in earlier chapters. However, the molecular mechanisms involved in sperm-uterine interactions are not studied in-depth in any mammalian species. Further, the uterus needs a defense mechanism against pathogens, sperm and semi-allogenic embryo as described previously. To achieve this, the uterus furnished with a well-developed immune system. Uterine endometrium develops a mucosal innate immunity as well as their ability to regulate the recruitment and action of immune cells of both innate and adaptive immunity [109]. The innate immune response relies on germ-line-encoded pattern recognition receptors (PRRs) to differentiate infectious non-self from non-infectious self [110].

Toll-like receptors (TLRs) are PRRs that can detect any changes in the micro-environment of the cells [111]. They are the membrane-spanning proteins accompanied by extracellular domains of leucine-rich repeats. Up to the date, 11 mammalian TLRs have been recognized with each receptor having its specific ligand [112]. In bovine endometrium, TLRs 1 to 10 have been identified [113]. Uterine epithelial cells express TLRs 1 to 7 and 9, and stromal cells express TLRs 1 to 4, 6, 7, 9 and 10 [113]. The TLR2 is significant in the bovine endometrium because of their ability to sense and respond to lipopeptides of Gram-positive bacteria that enter the uterus around the time of coitus or parturition [114]. Further, the TLR2/4 pathway contributes to the inflammatory cascades responsible for significant physiological processes such as ovulation [115], and fertilization [116].

We recently reported that sperm binding to monolayer cultures of bovine uterine epithelial cells (BUEC) induces an acute inflammatory response in the epithelium [24] by Toll-like receptor (TLR) signaling pathway via TLR2 [81]. Therefore, to investigate the mechanism involved in sperm-uterine interactions, as an initial step the involvement of TLR2 in sperm-uterine interaction and the inflammatory processes using the developed bovine endometrial explant culture model was evaluated.

2. Materials and methods

2.1. Incubation of sperm with bovine endometrial explants

The involvement of the TLR2 in sperm-uterine attachment and the inflammatory process was examined using TLR1/2 antagonist-CU-CPT22 (Merck, Darmstadt, Germany) [117]. The pre-ovulatory phase explants were pre-incubated with 1 μM antagonist in a 38.5˚C incubator with 5% CO_2 in humidified air for 30 min. Then the explants were incubated with 10^6 sperm/mL for 2 h. Explants without antagonists 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, Carlsbad, CA, USA), at -80˚C until mRNA extraction.

2.2. Observation of sperm behavior after incubation with endometrial explants

To evaluate the effect of TLR1/2 antagonist on sperm behavior, the pre-ovulatory phase endometrial explants were pre-incubated with 1 μM TLR1/2 antagonist in a 38.5˚C incubator with 5% CO_2 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. Then the rest of the protocol for observation of sperm behavior on endometrial explants was followed as described earlier. Briefly, 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 which contained 0.5 mL of 38.5˚C TALP, the explants 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.

2.3. Scanning electron microscopy (SEM)

To evaluate the effects of TLR1/2 antagonist on sperm entering uterine glands, the pre-ovulatory phase explants were pre-incubated with 1 μM TLR1/2 antagonist in a 38.5˚C incubator with 5% CO_2 in humidified air for 30 min. Then the explants were incubated with 10^6 cells/mL sperm for 30 min. Explants without TLR1/2 antagonist treatment served as controls. The experiment was repeated two times using endometrial explants from two different uteri.

The rest of the protocol for the SEM was followed as described earlier. Briefly, explants were gently washed in TALP and fixed in PB 10% formalin. 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, Tokyo, Japan) at an accelerating voltage of 5 kV.

2.4. 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 grayscale 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.

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

Isolation of RNA and analysis of mRNA by quantitative real-time PCR was performed as mentioned above. Briefly, endometrial explants were homogenized in TRIZOL reagent using an ultrasonic processor (Sonics and Materials Inc., CT, USA) under ice-cold conditions and further total RNA extraction was performed using TRIZOL reagent according to a protocol described previously [104]. The concentration and purity of extracted RNA were determined using NanoDrop Spectrophotometer (2000c, Thermo Scientific, Waltham, MA, USA) by the absorbance at 260 nm and the 260/280 nm ratios, respectively. After measurements, the RNA was stored at -80˚C in RNA storage solution (Ambion, Austin, TX, USA) until cDNA synthesis. The cDNA was synthesized following a

protocol described previously [73] and stored at -30˚C. Quantitative real-time PCR was carried out by a MiniOpticon (Bio-Rad Laboratories, Tokyo, Japan) using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Inc, USA). The amplification program was run with an initial activation step (15 min at 95˚C), followed by 40 cycles of PCR (15-sec denaturation at 95˚C, 15-sec annealing at 55-58˚C, and 30-sec extension at 72˚C). 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 gene expression were determined using the Delta-Delta comparative threshold method [105]. The primers used for each target are listed in table 1.

2.6. Statistical analysis

GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Students 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 ± standard error of the mean (SE_M). Data were considered to be statistically significant at *P<0.05, **P<0.01, ***P<0.001.

3. Results

3.1. TLR2 antagonists reduced sperm numbers in glands.

SEM images (Fig. 22A) and fluorescence video microscopy revealed that addition of TLR1/2 antagonist prior to adding sperm significantly reduced the sperm numbers in the glands ($p<0.001$) (Fig. 22B).

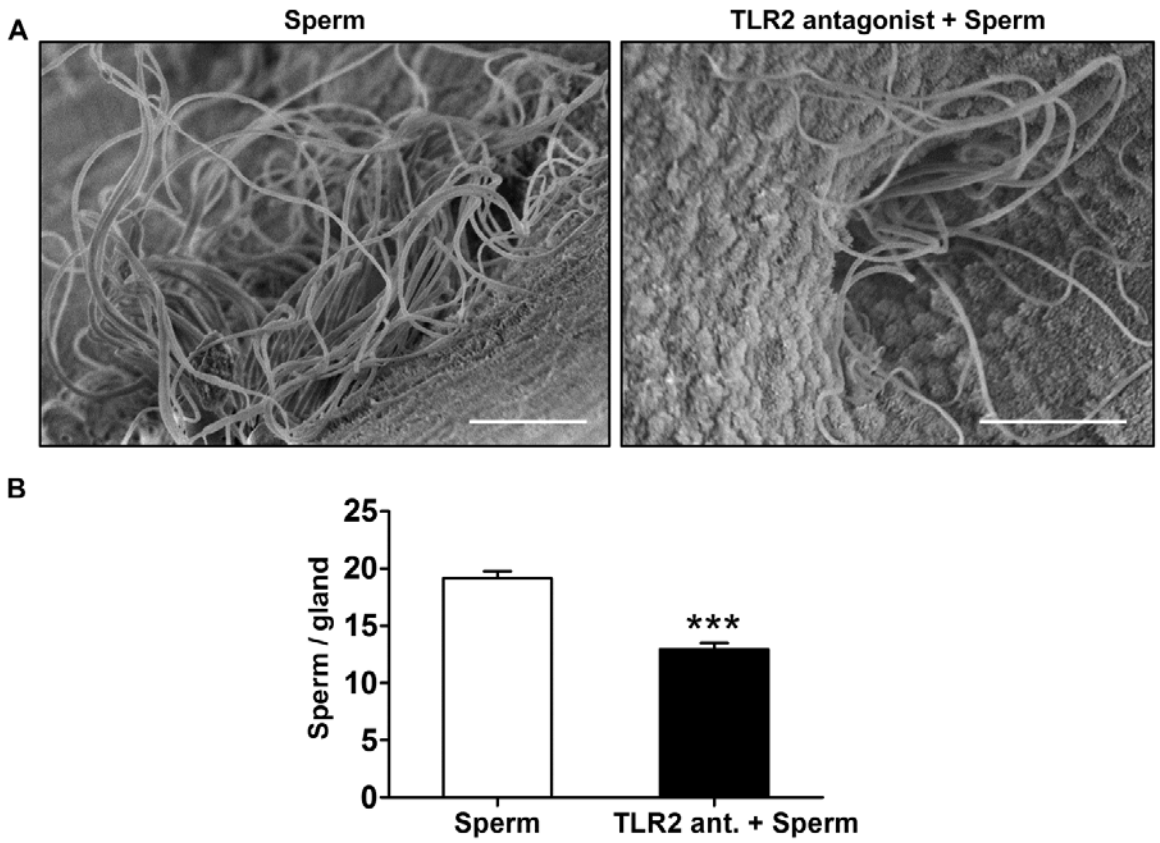

Figure 22. TLR2 antagonists reduced sperm numbers in uterine glands. **A.** SEM images of bovine endometrial explants that had been incubated with sperm +/- TLR1/2 antagonist. The pre-ovulatory phase endometrial explants were pre-incubated with 1 µM TLR1/2 antagonist for 30 min, followed by the addition of sperm and incubation for an additional 30 min. Bars=10 µm. **B.** The number of sperm/gland in explants incubated with sperm +/- TLR1/2 antagonist. Data are presented as mean ± SE_M of five independent experiments. For each treatment, sperm were counted in three uterine glands. ***p<0.001 denotes a significant difference.

3.2. TLR2 antagonist inhibited the gene expression.

The addition of TLR1/2 antagonist prior to adding sperm inhibited the increase of *TNFA* (Fig. 23A). 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. 23B).

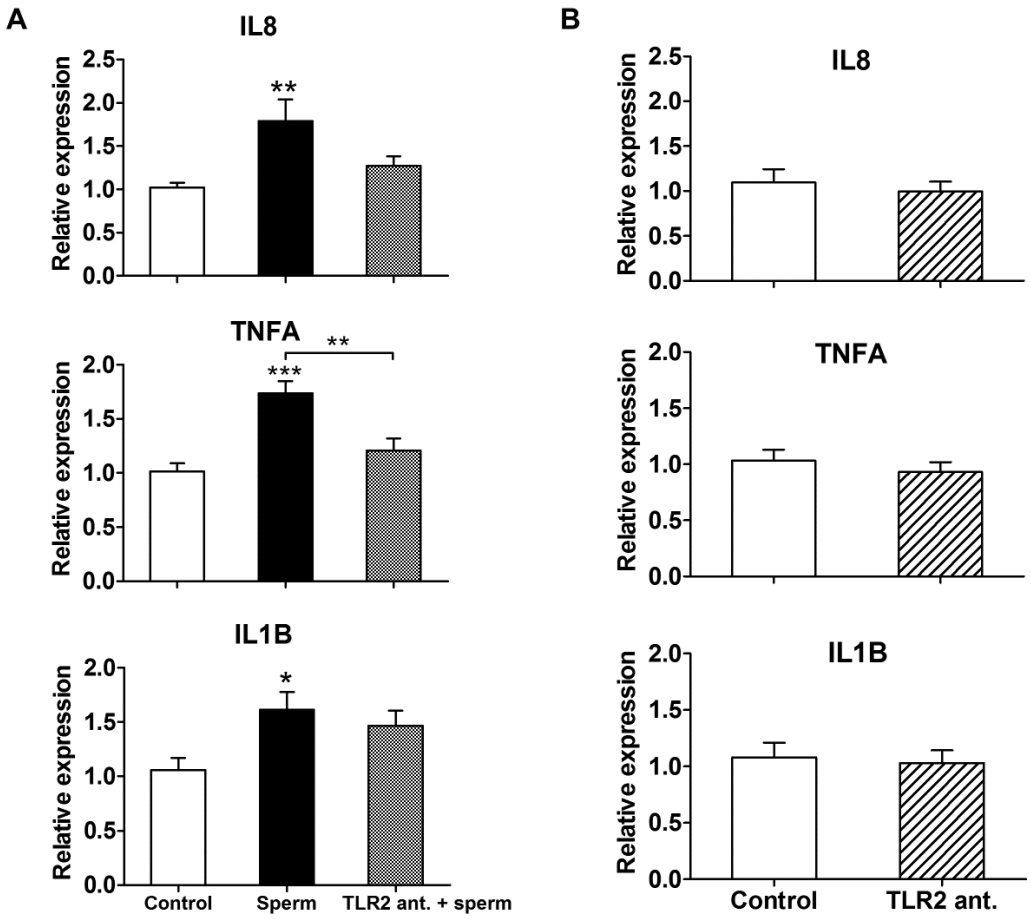

Figure 23. A. Relative mRNA expression of pro-inflammatory cytokines in pre-ovulatory phase explants incubated with sperm, sperm + TLR1/2 antagonist, and without sperm or antagonist for 2 h. **B.** Relative mRNA expression of pro-inflammatory cytokines in pre-ovulatory phase explants incubated with -/+ TLR1/2 antagonist for 2 h. Data are presented as mean ± SE_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.

4. Discussion

The present study using a co-incubation model of bovine endometrial explants and fresh sperm provide evidence that TLR2 involved in sperm-uterine interaction and immune responses. The addition of TLR2 blocker to the endometria explants reduced the sperm attachment to uterine glands and inhibited the increase of pro-inflammatory cytokines.

In a recent study using bovine endometrial epithelial cell culture model, we revealed that bovine endometrial epithelial cells respond to sperm through TLR2/4 signal transduction [81]. But the involvement of TLR2 in sperm-uterine interaction and immune responses not studied in detail.

As an initial step TLR2 was selected due to their ability to mediate inflammation at the interaction of sperm with cumulus-oocyte complexes during fertilization [116]. From the present data, the pre-incubation of bovine endometrial explants with TLR2 antagonists reduced the magnitude of sperm-induced inflammation. This observation supports the hypothesis that TLR2 involved in sperm-uterine immune responses. Further, the data provide evidence that TLR2 is involved in sperm-uterine interactions. Probably endogenous ligands mediate this interaction. There are many TLR2 endogenous ligands are reported such as, hyaluronan (HA) fragments [118], heat shock protein-70 (HSP70) [119], high mobility group box-1 protein (HMGB 1) [120], and biglycan [121]. Therefore, further investigations are necessary to find the possible sperm- TLR2 ligands involved in the interactions and immune responses using developed bovine endometrial explant culture model.

General discussion and conclusion

The 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 [89, 90]. 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.

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. 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. Glandular retention of sperm has been shown in histological sections of bovine uterine glands 24 h after AI [27], 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. The number of sperm trapped in the glands was roughly estimated as 0.5% of the sperm added to the explant culture wells. 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 the explant model with 8mm endometrial tissue disks, it is not possible to extrapolate the present data to the percentage of sperm that enter glands *in vivo*.

The present data indicate that sperm interaction with glandular epithelium triggers local uterine innate immune responses *via* the upregulation of pro-inflammatory cytokines. Sperm up-regulated mRNA expression of pro-inflammatory 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 [24]. Further, in the present study, synthesis of TNFA protein, a strong pro-inflammatory marker, was detected in the glandular epithelium. The immunohistochemistry results indicated that TNFA protein was expressed mainly in the glandular epithelium. The possibility that luminal epithelium is also involved in the inflammatory response cannot exclude completely, 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 role of luminal epithelium in activating the inflammatory cascade induced by sperm.

SEM images showed that PMNs were present in the glands. PMNs are known to rapidly enter the uterine lumen after insemination of cows [59], as well as of females of several other species of mammals [14, 52, 55, 56]. The present 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. 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 [54], goats [14], pigs [22], and rabbits [61]. PMN phagocytosis of sperm within uterine glands was reported in little brown bats, guinea pigs, horseshoe bats, rabbits, and pigs [25, 27, 28]. 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 [49].

The observations revealed that sperm entered and remained in the pre-ovulatory 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 [6, 8, 9, 106]. Although the physical attraction of sperm for

surfaces would tend to direct them into glands, as discussed above, the physical and/or chemical nature of the luteal phase secretions in the glands may have prevented them from doing so.

Previously our group reported that the addition of CU-CPT22, a TLR1/2 antagonist [117], 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 [81]. To check the involvement of TLR2 in the explant response to sperm, the effects of the antagonist on explant response to sperm were tested. Pre-incubation of uterine explants with antagonists 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 activation of the TLR2 signaling pathway by sperm.

It can be concluded that the developed 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 the 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. Further investigations are necessary to find the possible mechanism/chemoattractants from the uterine glands which attract the sperm and to explore the possible sperm- TLR2 ligands involved in the interactions and immune responses.

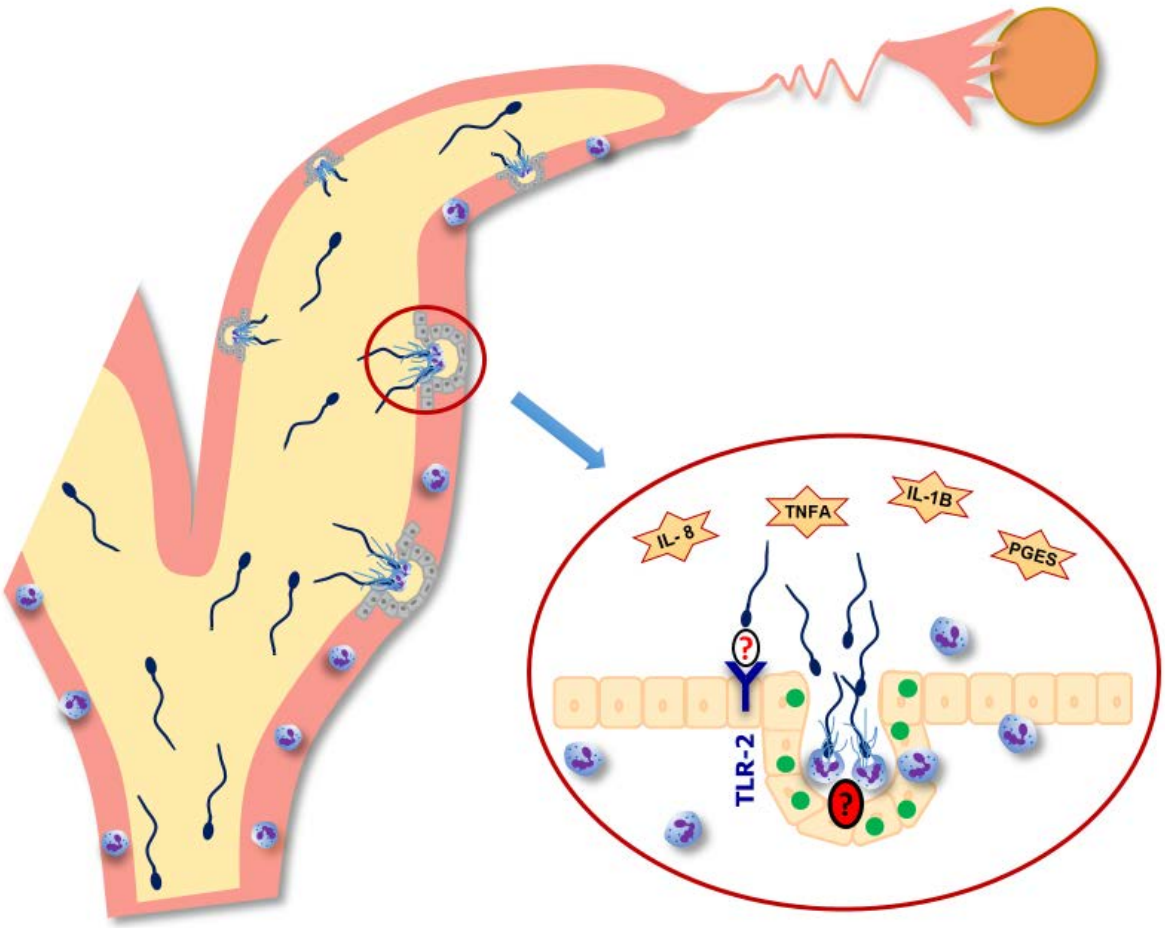

Figure 24. Illustration showing the findings of the study; Sperm entry into uterine glands triggers the innate immune responses. Green dots, TNFA protein expression in uterine glands. Black question mark, unknown mechanism/chemoattractants from the uterine glands which attract the sperm. Red question mark, unknown sperm-TLR2 ligand.

Summary

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.

Acknowledgement

First of all, I would like to express my sincere gratitude to my main supervisor Prof. Dr. Akio Miyamoto for accepting me as a PhD student and for his scholastic supervision throughout my study and research period. His motivational guidance, useful criticisms, and enthusiastic encouragement had inspired me to upgrade my thinking ability for getting new ideas in the research. I am really grateful to him for his valuable instructions during my study period, and also for taking care of my private life in Obihiro.

Besides my main supervisor, I would like to thank my co-supervisors Prof. Dr. Thakehiro Nishida, Animal and Food Hygiene, and Prof. Dr. Chiho Kawashima, Animal and Food Hygiene for their generous help, valuable guidance and suggestions throughout my research study.

Particularly I would like to provide my acknowledgements to my special advisor, Prof. Dr. Susan Suarez, Cornell University, Ithaca, USA, for her plentiful and constructive ideas in my research.

I am particularly grateful to Prof. Dr. Motoki Sasaki, Department of Veterinary Medicine for his support during Scanning Electron Microscopy and Immunohistochemical studies.

My grateful thanks are also extended to Prof. Dr. Masayuki Shimada, Hiroshima University for his kind support and suggestions in my study.

My sincere thanks go to Dr. Ken-ichi Takahashi, Tokachi bull center, Genetics Hokkaido Association, Japan, for providing us with bull fresh semen for the current study.

Further, I thank Anni Nurliani, Laboratory of Anatomy, for her technical support during Scanning Electron Microscopy and Immunohistochemical studies.

I am grateful to Dr. Mohamed Marey for his kind help during my research work. I am also thankful to Mr. Haruhisa (my Tutor) for his kind assistance in the laboratory and to my daily life. My sincere thanks also go to Morillo Vernadyn for help and assistance in my research. I would like to thank all PhD, Master, and undergraduate students of our laboratory for providing me with a friendly environment to work with.

Last but not least, I would like to express my deep gratitude to the Obihiro University of Agriculture and Veterinary medicine for providing me a scholarship for my doctoral study and also pleasant academic facilities.

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和 文 要 約

哺乳動物では、交配後に膨大な数の精子が子宮に侵入する。しかし多くの種では、ほんの数千の精子だけが卵管にたどり着く。精子が雌性生殖器を通過する際には、それらの上皮と同時に免疫細胞群とも相互に作用し合う。子宮に侵入した精子は上皮に作用し、それに続く一連の免疫反応は余分な精子、欠陥のある精子、死んだ精子などを子宮内から迅速に排除して、その後に起こる胚の着床に向けた準備を始めるために重要である。ウシにおいて、精子が子宮内膜とどのようにして相互に作用しあって免疫反応が誘導されるかについては、ほとんどわかっていない。私たちの研究グループは、これまでに精子がウシ子宮上皮細胞に結合すると、Toll 様受容体（TLRs）に関係するシグナル伝達経路が主に TLR2 を介して誘導されることを示した。しかしながら、上皮細胞の単層培養系は生体内の子宮組織とは大きく異なる。そこで、生体の子宮内で起こる精子–子宮の相互作用と免疫反応を観察するために、本研究では、*ex-vivo* での子宮組織培養系モデルを確立して、詳細について検証した。

第 1 章では、新鮮なウシの子宮上皮組織培養系を用いた *ex-vivo* モデルを開発した。健常な発情後の子宮から、排卵直前の卵胞側に接する子宮角の子宮小丘の間の子宮上皮組織を直径 8 mm のバイオプシーパンチで円型に切り取り、2 mm の厚さに調整した。この子宮小片を、10^6/ml の射出精液から洗浄して得た精子と共培養した。精子ミトコンドリアを JC1 で蛍光ラベルして、精子の子宮小片上での動態をビデオ観察した。同時に、走査型電子顕微鏡（SEM）で微細な観察も行なった。その結果、精子は子宮小片の表面を泳いですぐに子宮腺に侵入した。SEM 観察では、精子は子宮腺に頭部を入れ、尾部の多くは子宮腺開口部の外に出していた。以上から、ウシ子宮小片の *ex-vivo* モデルは、精子の子宮内で示す動態を詳細に観察することができ、ウシ精子が子宮内で示す動態とその後の子宮の免疫応答の検証に極めて有効なアプローチであることがわかった。

第 2 章では、このウシ子宮小片の *ex-vivo* モデルを用いて、精子が子宮腺に侵入して誘導する子宮の免疫応答について詳細を検証した。精子は 30 min 以内に好中球の強力なケモカインである *IL8* 遺伝子発現を増加させ、他の主要な炎症性サイトカインである *TNFA* や *IL1B* 遺伝子発現も *PGES* と *TLR2* 遺伝子発現と共に 2 h 以内に刺激した。さらに免疫組織化学による観察で、精子が侵入した子宮腺の内腔側の上皮細胞は、炎症性サイトカイン TNFA タンパクが発現していた。驚くことに、SEM 観察では精子が侵入した子宮腺に好中球が出現しており、精子を攻撃していた。これは、授精後に起きる精子貪食による子宮の清浄化の最初の現象かもしれない。熱で非動化した精子や、黄体期の子宮では、精子が子宮腺に侵入することはなく、これは発情–排卵期特有の現象であると考えられた。

第 3 章では、精子の子宮腺への侵入に始まる炎症反応が、TLR2 を介しているかを検証した。TLR2 アンタゴニストは、子宮腺に侵入する精子数を減少させ、炎症性サイトカイ

ンの *TNFA* 発現を抑制した。この事実から、精子–子宮の相互作用と免疫反応は、少なくとも一部は TLR2 を介することが示唆された。関係するシグナル経路については、さらなる詳細な検証が必要である。

以上の一連の研究結果から、ウシ子宮小片の *ex-vivo* モデルは精子–子宮の相互作用に始まる免疫反応（炎症作用）の起点となる現象を検証するのに有効であった。発見した子宮腺への精子の侵入と、そこから始まる子宮の炎症反応は、授精直後に始まる子宮の精子排除と清浄化カスケードの起点であり、その後の初期胚の子宮への受け入れに重要な現象であることが強く示唆された。